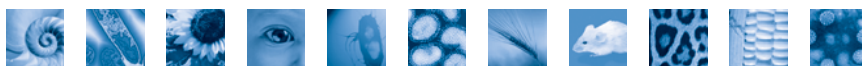




NimbleGen Arrays User's Guide

Sequence Capture Array Delivery

For life science research only.



HIGH - DEFINITION GENOMICS





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Sequence Capture Array Delivery

For life science research only.
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Notes

Chapter 1. Before You Begin

This user's guide describes the process for the capture of genomic DNA (gDNA) from specified regions of a genome using NimbleGen Sequence Capture arrays and amplification by ligation-mediated PCR (LM-PCR). Specifically, this user's guide describes quality control (QC) of gDNA samples, fragmentation of gDNA, annealing of amplification linkers, library preparation, LM-PCR on non-captured samples, hybridization of gDNA to and washing of the NimbleGen Sequence Capture array, elution of captured gDNA, LM-PCR of captured samples, and quantitative PCR (qPCR) assays to ensure successful capture (Figure 1).

At this time, you can set up the appropriate thermocycler reaction programs as detailed on page 22 (Chapter 4, Step 2.1), page 36 (Chapter 6, Step 2.2), page 60 (Chapter 10, Step 3.2), and page 63 (Chapter 11, Step 1.5).

What's New?

- A new, preferred method for eluting captured DNA from NimbleGen 385K and 2.1M arrays using sodium hydroxide solution has replaced the previous elution using Elution Reagent. Refer to Chapter 9.

The previous method for the elution of captured DNA using Elution Reagent is provided as an alternative method. Refer to Appendix E.
- The captured LM-PCR and clean-up processes were updated for use with the new sodium hydroxide elution method. Refer to Chapter 10.
- Chapter 12, 454 Sequencing of the Sequence Capture Library, in version 3.0 of the user's guide has been condensed and moved to Appendix B, Sequencing with Next Generation Platforms, in this user's guide. Instructions for sequencing with 454 GS FLX Standard chemistry have been discontinued in favor of GS FLX Titanium chemistry.
- Recommendations for performing high-throughput Sequence Capture experiments are also provided. Refer to Appendix F.

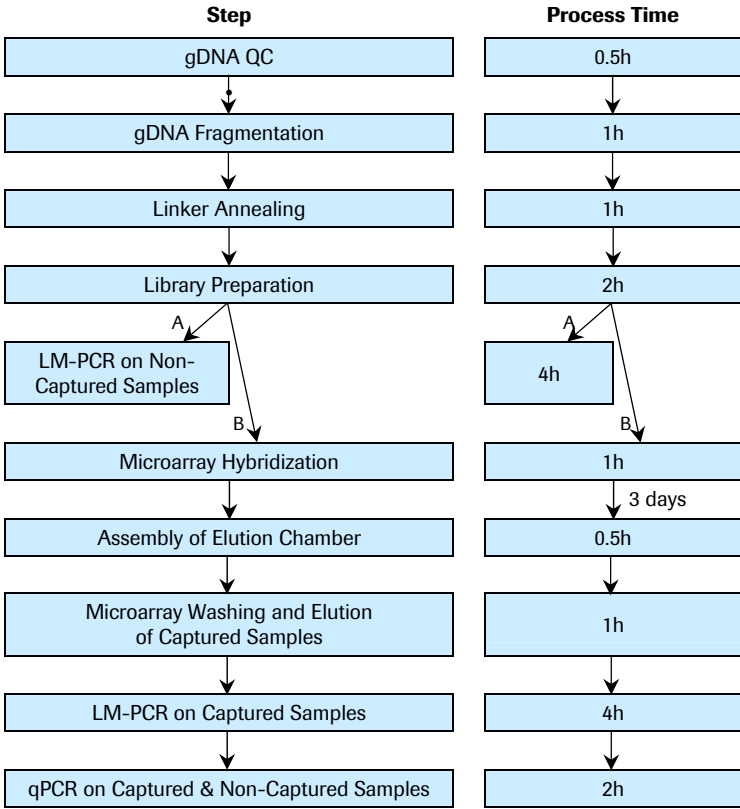


Figure 1: Workflow for NimbleGen Sequence Capture Experiments. Steps in the process and estimated time for each step, based on the processing of one array, are shown in the boxes. Incubation times are indicated beneath the appropriate process times.

Components Supplied

Component	Description
NimbleGen Arrays	As ordered
NimbleGen Mixers	X1 mixer is provided with the 385K array; HX1 mixer is provided with the 2.1M array.
Mixer Port Seals	Port seals are provided with both X1 and HX1 mixers
Elution Chamber (ES1 or EL1)	Disposable apparatus used with the NimbleGen Elution System for elution of captured DNA from 385K arrays (ES1) or 2.1M arrays (EL1)
NimbleGen Arrays User's Guide: Sequence Capture Array Delivery	This booklet
CD/DVD	Design .gff and .bed files, visualization software, and user documentation are included

Microarray Storage

NimbleGen Sequence Capture microarrays are sealed with desiccant and can be stored at room temperature until ready to use.

Store opened NimbleGen microarrays in a desiccator at room temperature until ready to use.

Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- Perform all centrifugations at room temperature unless indicated otherwise.

Required Equipment, Labware & Consumables

You assume full responsibility when using the equipment, labware, and consumables described below. These protocols are designed for the specified equipment, labware, and consumables.

NimbleGen Equipment

Choose between the 4- or 12-bay NimbleGen Hybridization Systems.

Equipment	Supplier	Process Quantity	Item Number
NimbleGen Hybridization System 4*	Roche NimbleGen	4 slides	05223652001 (110V)
			05223687001 (220V)
NimbleGen Hybridization System 12*	Roche NimbleGen	12 slides	05223679001 (110V)
			05223695001 (220V)
NimbleGen Elution System	Roche NimbleGen	1 slide	05329752001

* NimbleGen Hybridization Systems include an accessory kit that contains Precision Mixer Alignment Tool (PMAT), Mixer Disassembly Tool, Mixer Brayer, System Verification Assemblies, replacement O-rings, and forceps.

General Laboratory Equipment

Equipment	Supplier	Item Number
NimbleGen Compressed Gas Nozzle	Roche NimbleGen	05223628001
Sonicator 4000	Misonix	S-4000
MicroAmp Optical 96-Well Reaction Plate	Applied Biosystems	N801-0560
AlumaSeal II Film (1.5ml, pressure foil seals)	Phenix Research Products	LMTALUMAI
Nitrogen or Argon Tank with 1 - 125psi regulator	VWR	55850-700
GS Nebulizers Kit (for nebulization only)	Roche Applied Science	05160570001
Conical Tubes (15ml sterile screw cap)	Multiple Vendors	
Syringe Filter (0.2µ, surfactant free, cellulose acetate - SFCA)	Multiple Vendors	
Dynal MPC-S (6 x 1.5ml tube holder)	Invitrogen	120-20D

Equipment	Supplier	Item Number
Desiccator	<i>Multiple Vendors</i>	
Electrophoresis System	<i>Multiple Vendors</i>	
Heat Block (capable of temperatures to 120°C)	<i>Multiple Vendors</i> (Recommend the 6-bay digital hot block from VWR, #126210100)	
Microcentrifuge with Multiplate Adaptors (12,000 x g capability)	<i>Multiple Vendors</i>	
Spectrophotometer	NanoDrop	ND-1000
Bioanalyzer 2100	Agilent	
LightCycler 480 Instrument II	Roche Applied Science	05015243001 (384-well version) -or- 05015278001 (96-well version)
Genome Sequencer FLX Instrument	Roche Applied Science	04896548001
SpeedVac (1.5ml tubes)	Thermo Savant	
Thermocycler	<i>Multiple Vendors</i>	
Vortex Mixer	<i>Multiple Vendors</i>	
QIAvac 24 Vacuum Manifold	Qiagen	19413
Vacuum Pump	Qiagen	84010

Consumables & Accessories from Roche NimbleGen

Component	Package Size / Process Quantity	Item Number
NimbleGen X1 Mixer for 385K arrays (includes mixer port seals)	10 mixers	05223725001
NimbleGen HX1 Mixer for 2.1M arrays (includes mixer port seals)	10 mixers	05223741001
NimbleGen Sequence Capture Hybridization Kit	60 hybridizations for 385K arrays 25 hybridizations for 2.1M arrays	05340721001
NimbleGen Sequence Capture Wash and Elution Kit	15 washes and elutions	05340730001
Elution Chamber ES1 ¹	1 chamber & 1 gasket	05329809001
Elution Chamber EL1 ¹	1 chamber & 1 gasket	05329817001
Elution System Retaining Ring (only order if replacement is needed) ²	1 retaining ring	05329787001

1 An Elution Chamber (ES1 for 385K or EL1 for 2.1M), included with a NimbleGen Sequence Capture array, can be purchased separately. Elution chambers and gaskets are disposable and intended for a single use.

2 A NimbleGen Elution System Retaining Ring, included with a NimbleGen Elution System, can be ordered as a replacement part.

Contents of NimbleGen Sequence Capture Kits

Kit	Contents
NimbleGen Sequence Capture Hybridization Kit	■ 2X SC Hybridization Buffer* ■ SC Hybridization Component A
NimbleGen Sequence Capture Wash and Elution Kit	■ 2X Stringent Wash Buffer ■ 10X SC Wash Buffer I ■ 10X SC Wash Buffer II ■ 10X SC Wash Buffer III ■ Elution Reagent

* SC = Sequence Capture

Consumables from Other Vendors

Choose sonication or nebulization for fragmentation of genomic DNA.

Component	Supplier	Package Size	Item Number
100X BSA	NEB	10mg/ml	B9001S
100mM ATP (ribonucleotide)	Roche Applied Science	40μmol	11140965001
0.5M EDTA, pH 8.0	Sigma Aldrich	100ml	E7889
1M MgCl ₂	Sigma Aldrich	100ml	M1028
1M Tris HCl, pH 7.8	Sigma Aldrich	1 liter	T-2913
1M Tris HCl, pH 7.5 (for nebulization only)	Fisher Scientific	500ml	BP1757-500
Glycerol (for nebulization only)	Multiple Vendors		
5M NaCl	Sigma Aldrich	250ml	71386
70% Ethanol	Multiple Vendors		
Tween 20	Sigma Aldrich	100ml	P7949
Compressed Nitrogen or Argon Gas (for cleaning array surface)	Multiple Vendors		
CP100 Pipette Tips	Gilson	192 tips	F148414
		960 tips	F148314
Microfuge Tubes (1.5ml)	Ambion	500 tubes	12400
Pipettor Basin (multichannel, 100ml)	VWR	200 basins	82026-356
Disposable Forceps	Cole-Parmer	100 forceps	EW-06443-20
Washing Tubes	Fisher Scientific	1,000 tubes	05-553-4
T4 DNA Polymerase & Buffer 2	NEB	3U/μl	M0203L
DNA Clean and Concentrator-25 Kit	Zymo Research	50 preps	D4033
T4 Polynucleotide Kinase	NEB	10U/μl	M0201L
T4 DNA Ligase	NEB	400U/μl	M0202L
AMPure DNA Purification Beads	Agencourt	5ml	A50850

Component	Supplier	Package Size	Item Number
Water (reagent grade, ACS, nonsterile, type 1)	VWR	2.5 gallon	RC915025
Bioanalyzer 2100 DNA Chip 7500 Kit	Agilent	25 chips	5067-1506
Bioanalyzer 2100 DNA Chip 1000 Kit	Agilent	25 chips	5067-1504
10X ThermoPol Reaction Buffer	NEB	4 x 1.5ml	B9004S
25mM dNTP	Epicentre	10μmoles	059104
Taq DNA Polymerase	Qiagen	5U/μl	201203
PfuTurbo DNA Polymerase	Stratagene	2.5U/μl	600252
LightCycler 480 Multiwell Plate 384 (with sealing foils)	Roche Applied Science	5 x 10 plates	04729749001
LightCycler 480 SYBR Green I Master (2X Mix)	Roche Applied Science	5 x 1ml	04707516001
Human Cot-1 DNA	Invitrogen	1mg/ml	15279-011
Water (PCR-grade)	Roche Applied Science	25 x 1ml	03315932001
10bp DNA Step Ladder	Promega	0.65μg/μl	G4471
10M Sodium Hydroxide	Sigma-Aldrich	100ml	72068
Acetic Acid, Glacial	Fisher Scientific	500ml	BP1185
MinElute PCR Purification Kit (250)	Qiagen	250 columns	28006
QIAquick PCR Purification Kit	Qiagen	50 columns	28104

Oligonucleotide Consumables

Component	Supplier	Concentration	Sequence
gSel3 DNA Oligo	IDT	4,000μM	5' - CTC GAG AAT TCT GGA TCC TC - 3'
gSel4-P _i DNA Oligo	IDT	4,000μM	5' - Phos/GAG GAT CCA GAA TTC TCG AGT T - 3'
qPCR Oligo NSC-0237 forward*	IDT	2μM	5' - CGC ATT CCT CAT CCC AGT ATG - 3'

Component	Supplier	Concentration	Sequence
qPCR Oligo NSC-0237 reverse*	IDT	2μM	5' - AAA GGA CTT GGT GCA GAG TTC AG - 3'
qPCR Oligo NSC-0247 forward*	IDT	2μM	5' - CCC ACC GCC TTC GAC AT - 3'
qPCR Oligo NSC-0247 reverse*	IDT	2μM	5' - CCT GCT TAC TGT GGG CTC TTG - 3'
qPCR Oligo NSC-0268 forward*	IDT	2μM	5' - CTC GCT TAA CCA GAC TCA TCT ACT GT - 3'
qPCR Oligo NSC-0268 reverse*	IDT	2μM	5' - ACT TGG CTC AGC TGT ATG AAG GT - 3'
qPCR Oligo NSC-0272 forward*	IDT	2μM	5' - CAG CCC CAG CTC AGG TAC AG - 3'
qPCR Oligo NSC-0272 reverse*	IDT	2μM	5' - ATG ATG CGA GTG CTG ATG ATG - 3'

* These oligos are used in qPCR analysis, described in Chapter 11.

Consumables for the Genome Sequencer FLX Instrument

Component	Supplier	Package Size	Item Number
GS FLX Standard DNA Library Preparation Kit	Roche Applied Science	10 library preparations	04852265001
GS FLX Titanium General Library Preparation Kit	Roche Applied Science	10 library preparations	05233747001
Tris-EDTA, 1X (pH 8.0)	Fisher Scientific	1 liter	BP2473-1
Acetic Acid, Glacial Aldehyde-free Sequencing	Fisher Scientific	500ml	BP1185-500
Sodium Hydroxide Solution (5.0M)	Sigma Aldrich	150ml	S8263-150ML
MinElute PCR Purification Kit (250)	Qiagen	250 purifications	28006

Technical Support

If you have technical questions, contact your Roche Account Manager or Roche Microarray Technical Support. Go to www.nimblegen.com/arraysupport for contact information.

Chapter 2. Sample Requirements and QC

Chapter 2 describes sample requirements and the QC protocol for NimbleGen Sequence Capture experiments.

Sample Requirements

Twenty micrograms of high-quality, high molecular weight, unamplified gDNA are suggested to perform a Sequence Capture experiment with a single 385K or 2.1M array.

- DNA samples should have a concentration of approximately 250ng/μl to 500ng/μl in nuclease-free water or TE buffer.
- Visual inspection (via agarose gel electrophoresis) of DNA should show little evidence of degradation. RNA contamination interferes with the quantification of DNA. Samples can be treated with RNase to eliminate residual RNA.
- Samples with an $A_{260}/A_{280} \geq 1.8$ and $A_{260}/A_{230} \geq 1.9$ are ideal.

***Note:** A low A_{260}/A_{280} or A_{260}/A_{230} ratio is indicative of low DNA purity. If the minimum ratio value listed above is not met, Roche NimbleGen recommends that an additional precipitation be performed before proceeding with fragmentation of gDNA.*

Sample QC

1. Transfer 200ng of each sample to a sterile microcentrifuge tube. Store the remainder of your sample at -20°C until required for fragmentation.
2. Load 200ng of each sample onto a 1% agarose gel and analyze by electrophoresis with appropriate molecular weight markers to ensure that the gDNA is not degraded or displays RNA contamination (Figure 2).

Important: Genomic DNA should appear as a single prominent band greater than 12kb. If the sample appears as more than one band or as a smear, the DNA may be degraded or have a contaminant that could affect the library preparation. RNA contamination will result in a smear less than 200bp.

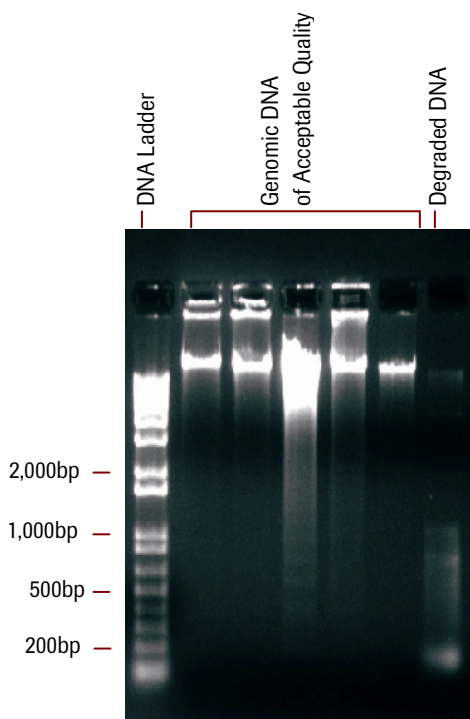


Figure 2: Example of Agarose Gel Electrophoresis Displaying Acceptable and Degraded gDNA Samples

Chapter 3. gDNA Fragmentation

Chapter 3 describes two different processes for fragmenting gDNA into a size range suitable for use in NimbleGen Sequence Capture experiments.

Typically, there are three options for genomic fragmentation: sonication, nebulization, or hydrodynamic shearing. This user's guide provides details for nebulization and sonication because these two options are less expensive than hydrodynamic shearing.

Nebulization is preferred because it produces a more uniform population of molecules (e.g. more centered with respect to the mode fragment size) and is very reproducible between different research groups. However, nebulization suffers from greater sample loss and is a lower throughput method compared to sonication.

Sonication is less preferred because the protocols are subject to apparatus drift (i.e. variance in shearing), and it requires more monitoring. However, sonication results in less sample loss and it allows for single tube library construction. The sonication protocol must be empirically established for each apparatus and sonicator type: plate versus probe. It is critical to establish a protocol where the mode fragment size is greater than 300bp. The plate format allows for the highest throughput.

References

- Sonicator 4000 Ultrasonic Liquid Processor Operation Manual (Misonix)
- Microplate Horn Operating Instructions (Misonix)
- Sonicator Series Application Notes AN-11 (Misonix)
- Microplate Heat Sealer Manual (4Seal) (Bioeke)
- GS DNA Library Preparation Kit Protocol (Roche Applied Science)
- DNA Clean & Concentrator-25 Kit Instructions (Zymo Research)

► **Nebulization: Preferred Fragmentation Process**

Nebulization is the preferred fragmentation technique over sonication. The instructions below describe the recommended use of the GS Nebulizers Kit (Roche Applied Science). If you would rather perform the sonication technique, skip this section and proceed directly to “Sonication: An Alternative Fragmentation Process.”

Step 1. Nebulize the Sample

1. Prepare 10ml of Nebulization Buffer by adding the following to a 15ml screw-cap conical tube:

Nebulization Buffer	
Glycerol	5.31ml
1M Tris-HCl (pH 7.5)	0.37ml
0.5M EDTA (pH 8.0)	0.11ml
VWR water	4.21ml
Total	10ml

2. Mix the Nebulization Buffer by vortexing.

Note: Store at -15°C to -25°C for a maximum of 1 month. Do not use if expiration date has passed.

3. Set the regulator on the nitrogen or argon tank to deliver gas at 45psi.
4. Pipette the following into the Nebulizer cup (Figure 3). Mix by pipetting up and down.

Component	
gDNA (in VWR water or TE buffer)	20µg
Water	To a total volume of 200µl (gDNA + water)
Nebulization Buffer (Step 1.1)	400µl
Total	600µl

5. Assemble the Nebulizer apparatus as shown in Figure 3.

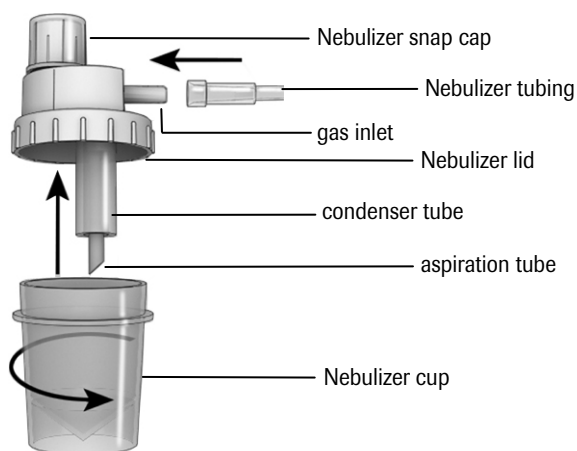


Figure 3: Diagram of the Nebulizer Apparatus

6. Position a Nebulizer snap cap over the Nebulizer chimney (not labeled in Figure 3; but it is the protrusion on top of the Nebulizer lid), and press firmly until the cap snaps into position. Ensure the Nebulizer snap cap is snug against the top of the lid.
7. Affix a condenser tube (soft rubber, in its own envelope) around the aspiration tube (molded into the Nebulizer lid). Ensure that the condenser tube is snug against the aspiration tube. Be careful not to rotate the aspiration tube.
8. Tightly screw the cup (with the DNA mixture in it) onto the Nebulizer lid (with condenser tube affixed, Steps 1.6 and 1.7), and connect one end of the Nebulizer tubing onto the gas inlet of the assembled Nebulizer apparatus (Figure 3).

Note: To contain sample molecules and prevent library cross-contamination, conduct nebulization using an externally vented hood (chemical safety hood is acceptable).

9. Connect the loose end of the Nebulizer tubing to the nitrogen or argon tank.
10. Direct 45psi of gas for 1 minute through the Nebulizer apparatus.

Note: Nebulization is governed largely by pressure rather than time. Less pressure will make the fragments larger, while more pressure will make the fragments smaller.

Warning! Do not increase the pressure without a means to contain the Nebulizer snap cap. High pressure can cause the Nebulizer apparatus to disassemble, generating a safety hazard. Nebulization without the Nebulizer snap cap can be performed but increases the amount of sample loss.

- 11.** Turn off the gas flow, wait 30 seconds, and then remove the Nebulizer tubing.
- 12.** Tap the Nebulizer apparatus on the bench top to collect the dispersed liquid. If necessary, tap the apparatus repeatedly to collect the residual material.

Important: This step is key to efficient recovery of nebulized DNA.

Note: Liquid can also be spun down from the aspiration tube by manual centrifugation with the waving of your arm.

- 13.** Unscrew the cap and use a pipette to measure the remaining volume of DNA.

Step 2. Clean up the Sample

- 1.** Follow the instructions provided with the DNA Clean & Concentrator-25 Kit (Zymo Research) with slight modifications (listed below in Steps 2.3 - 2.10).
- 2.** Measure the volume recovered from the Nebulizer apparatus.
- 3.** Add 2 volumes of DNA Binding Buffer Zymo Kit (~800µl) to the Nebulizer cup and mix well.
- 4.** Transfer 750µl of the mixture to a provided Zymo-Spin Column in a collection tube.
- 5.** Centrifuge at $\geq 10,000 \times g$ for 1 minute. Discard the flow-through.
- 6.** Transfer the remaining mixture to the same Zymo-Spin Column.
- 7.** Centrifuge at $\geq 10,000 \times g$ for 1 minute. Discard the flow-through.
- 8.** Add 200µl of Zymo Wash Buffer to the column. Centrifuge at $\geq 10,000 \times g$ for 1 minute.

9. Repeat Step 2.8.
10. Add 80µl of VWR water or 1X TE buffer directly to the column matrix. Transfer the column to a new 1.5ml microfuge tube and centrifuge at $\geq 10,000 \times g$ (maximum rpm) for 1 minute to elute the DNA.

Step 3. Analyze Results

1. Prepare an Agilent Bioanalyzer 2100 DNA Chip 7500 according to the manufacturer's instructions.
2. Load 1µl of sample into each well of the chip (up to 11 wells).
3. Load 1µl of VWR water as a blank well.
4. Run the chip using a DNA 7500 smear or ladder assay.
5. Overlay the results with the ladder (Figure 4).

***Note:** The ideal molecular population should be normally distributed with a mode between 300bp and 500bp; closer to 500bp is preferred. If there is variation of 20% or more in mode fragment size or some samples exhibit bimodal fragment distributions, consider repeating Step 1, Nebulize the Sample.*

The fragments are now ready for polishing (Chapter 5) and can be stored at -20°C.

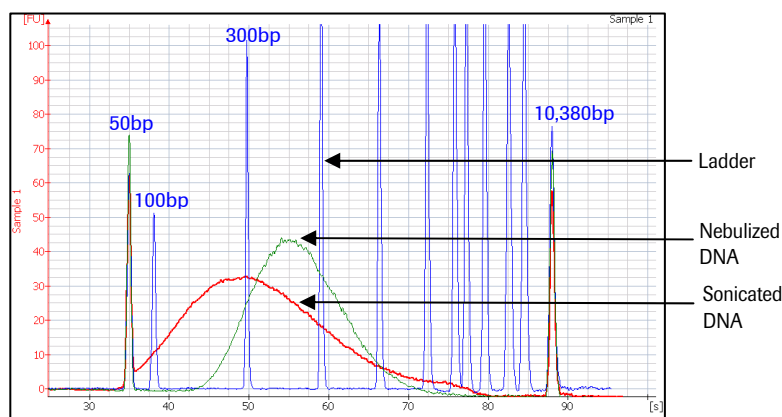


Figure 4: Example Agilent Bioanalyzer 2100 DNA Chip 7500 Run

- Sonicated DNA: 50bp - 1,500bp; mode at 300bp
- Nebulized DNA: 150bp - 1kb; mode is 400bp
- Ladder: 50*, 100*, 300*, 500, 700, 1,000, 1,500 ... 10,380*

* These peaks are labeled in the ladder trace for reference purposes only.

► Sonication: Alternative Fragmentation Process

The instructions below describe the recommended processes required using the Sonicator 4000 Ultrasonic Liquid Processor (Misonix) for 96-well plates. If using a probe sonicator or other type of equipment, adjust the processes to obtain equivalent results.

Step 1. Prepare Samples

1. With a marker, place a dot on the A1 corner of the microplate. This dot provides a visual reminder for plate orientation.
 2. Add 20µg of each sample to the plate, pipetting one sample per well.
 3. Bring each well up to 80µl total volume with VWR water.
 4. Seal the plate using AlumaSeal II Film.
 5. Place the plate in a PCR rack. Let sit until the plate reaches room temperature.
 6. Create a balance plate for the centrifuge.
 7. Centrifuge the plate for a couple of seconds to bring all contents to the bottom of the wells, eventually increasing the speed up to ~2,000rpm. Press the Stop button when the centrifuge reaches 2,000rpm.
-

Step 2. Sonicate the Sample

Note: For safety purposes, ensure that the sonicator's lid is closed and hearing protection is worn during sonication.

1. Empirically determine a sonication protocol that yields molecules with a molecular weight from 100 - 700bp, with a mode between 300 - 500bp.
2. Adjust and set sonicator settings (based on your protocol). Turn on the sonicator.
3. Place the microplate in the microplate horn. Fill the horn with water until the well volume is completely submerged.

4. Secure the microplate into place by applying tape to the top of the microplate and side of the microplate horn.
 5. Pack ice around the microplate while carefully monitoring the liquid level.
Note: Due to the possibility of reducing the transmission of ultrasonic energy, ensure that ice does not get under samples.
 6. Close the lid. Press the Start button. When sonication is complete, remove the plate from the microplate horn.
 7. Centrifuge the plate for a couple of seconds to bring all contents to the bottom of the wells, eventually increasing the speed up to ~2,000rpm. Press the Stop button when the centrifuge reaches 2,000rpm.
-

Step 3. Analyze Results

Analyze results from sonication using the same process as for nebulization. Refer to page 17 for detailed instructions.

Note: The ideal molecular population in Figure 4 should be normally distributed with a mode between 300bp and 500bp; closer to 500bp is preferred. If there is variation of 20% or more in mode fragment size or some samples exhibit bimodal fragment distributions, consider repeating Step 2, Sonicate the Sample.

Notes

Chapter 4. Linker Annealing

Chapter 4 describes the process for the annealing of linkers for library preparation (Chapter 5). This protocol details the annealing and validation of sufficient linker material for the construction of 5 Sequence Capture libraries. Adjust the volumes specified accordingly when constructing more than 5 libraries.

Reference

IDT website (www.idtdna.com)

Step 1. Prepare Linkers and Master Mix

1. Prepare the Oligo Annealing Buffer (OAB) by adding the following in a 15ml screw-cap, conical tube:

Oligo Annealing Buffer (OAB)	
1M Tris-HCl (pH 7.8)	100µl
0.5M EDTA (pH 8.0)	20µl
5M NaCl	100µl
VWR water	9.78ml
Total	10ml

2. Sterilize by filtration with a 0.2µ syringe filter.
3. Open a web browser and access the IDT Scitools resuspension calculator: www.idtdna.com/analyzer/Applications/resuspensioncalc/.
4. Enter the *QUANTITY* of oligo obtained from the synthesis of gSel3 (nmole) on your synthesis report sheet from IDT.
gSel3 = 5' - CTC GAG AAT TCT GGA TCC TC - 3'
5. Adjust the dropdown (OD260 is the default setting) so that the units match (nmole).

6. Click the **Single-stranded DNA, 33 µg/OD260** button.
7. Enter 4,000 into the *Final Concentration* field.
8. Leave the dropdown at the default setting of µM (µmole/liter or pmole/µl).
9. Click the **CALCULATE** button to display the amount of OAB necessary to resuspend the oligo.
10. Briefly centrifuge the lyophilized gSel3 oligo to ensure the pellet is at the bottom of the tube.
11. Add the indicated volume of OAB to the gSel3 tube, vortex, and spin down. Incubate at room temperature for 30 minutes.

Note: If the program indicates to add less than 10µl of OAB, adjust the FINAL CONCENTRATION to 2,000µM. If the program still indicates to add less than 10µl, adjust the FINAL CONCENTRATION to 500µM.

12. Repeat Steps 1.2 - 1.11 for gSel4-P_i. Ensure that both primers are brought to the same concentration.

gSel4-P_i = 5' - Phos/GAG GAT CCA GAA TTC TCG AGT T - 3'

13. In 0.2ml strip tubes, mix 5µl of 4,000µM gSel3 with 5µl of 4,000µM gSel4-P_i. Prepare as many tubes as possible with the synthesized primers. Each tube now contains 2,000µM of each linker.

Note: If each tube contains 2,000µM of each linker, it contains sufficient annealed linkers for use in the construction of 5 Sequence Capture libraries. Increase the amount of primers used for annealing accordingly when constructing more than 5 libraries.

Note: If it was necessary to decrease the primer concentration to get the calculator to yield a volume larger than 10µl, this concentration would be lower than 2,000µM. In the case of 2,000µM primer concentration, the mixed linker concentration equals 1,000µM of each linker.

Step 2. Anneal Primers to Make Linkers

1. Program a thermocycler (with a heated lid) with the following conditions (name the program "Linkers"):

- 95°C for 5 minutes
 - Ramp cool 0.1°C per second to 12°C
 - Hold at 12°C
2. Place the strip tube(s) with the primers into the thermocycler and run the “Linkers” program.
 3. When the thermocycler program is completed, remove the strip tube(s) and place on ice.
 4. Add 30µl of OAB to one 0.5ml PCR strip tube.

Note: This dilution creates a 500µM working stock of linkers. If it was necessary to alter the primer concentrations, dilute the linkers to 500µM accordingly.

5. Store linkers at -20°C.

Note: The linkers may be stored in their concentrated form or as a working stock. Each working stock tube contains sufficient linker to create 5 libraries.

Step 3. Quality Control Linkers

The linkers should be evaluated for their robustness in ligation reactions. Test ligations should be set up and analyzed by agarose gel electrophoresis.

1. Pour a 4% 1X TAE agarose gel and leave 2 lanes open for each set of annealed linkers.
2. Label two 0.2ml strip tubes (or plate wells) for preparation of Ligation Master Mix as follows: “+” or “-”
3. Prepare two Ligation Master Mixes:

“+” Master Mix (per linker set evaluated)	
NEB Buffer 2 (10X)	1µl
100mM ATP (ribonucleotide)	1µl
VWR water	5µl
NEB Ligase	1µl
Total	8µl

“-” Master Mix (per linker set evaluated)	
NEB Buffer 2 (10X)	1µl
100mM ATP (ribonucleotide)	1µl
VWR water	6µl
Total	8µl

- Pipette 2µl of each 500µM linker working stock to be evaluated into 2 wells/tubes (i.e. 1 well/tube for the “+” and 1 tube/well for the “-”).
- Add 8µl of the “+” Master Mix into each “+” sample to be evaluated.
- Repeat for the “-” Master Mix and “-” sample to be evaluated.
- Incubate at room temperature or 25°C in a thermocycler (heated lid is not necessary) for 45 minutes.
- Run each “+” and “-” pair (i.e. the same linker set) beside each other on the prepared agarose gel.

Important: Remember to load suitable molecular weight markers (i.e. 10bp ladder).

Note: ATP is very sensitive to multiple freeze-thaw cycles and has a short half-life at room temperature. It is highly recommended to aliquot the ATP (ribonucleotide) into 10µl working stocks and store at -20°C until needed. Treat all such aliquots as single-use reagents.

- Evaluate the agarose gel with ethidium fluorescence. Determine how much linker complex is at 20bp and how much linker complex is at 40bp.

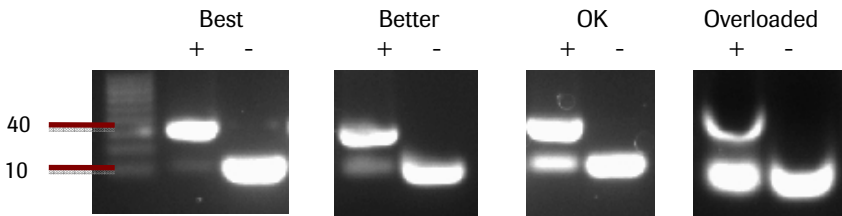


Figure 5: Examples of Linker Quality Control. Ideally most of the linker complex will be present at 40bp (i.e., annealed and ligated). Linkers are considered acceptable if $\geq 50\%$ of the linker complex is located at 40bp after the ligation test (notice very little un-ligated linker is located at 20bp in the “Best” test ligation, and $\sim 40\%$ un-ligated linker is located at 20bp in the “OK” ligation). A complete failure of linker ligation would appear as both “+” and “-” lanes looking identical to each other and located at 20bp.

***Note:** The 2 lanes labeled “Overloaded” do **not** represent a ligation failure. Instead they represent E-Gel lanes that are overloaded, as evidenced by the noticeable smile-shaped bands. The DNA-intercalating dye appears to be sensitive to depletion, such that successful ligations appear to have failed because there is not enough dye present to stain both the un-ligated and ligated adapters. When the same DNA in the “+” and “-” lanes was diluted, the results resembled the passing results in the “Better” panel. If you are using E-Gel systems instead of traditional agarose gels, we recommend a 1:10 dilution of the DNAs to prevent this kind of overloading artifact.*

- 10.** If > 50% of the complex is un-ligated (approximately equal to the “-” ligase control reaction), the linkers should be re-annealed and repeated for quality control. If the re-annealed linkers continue to fail quality control, consider ordering another synthesis of gSel4-P_i and/or setting up a kinase reaction with only gSel4 as the substrate.

***Note:** Efficient ligation requires a 5' phosphate group on gSel4, which can be added enzymatically to boost the ligation efficiency of the resulting linkers if the chemical synthesis of the 5' phosphate was not optimal.*

Notes

Chapter 5. Library Preparation

Chapter 5 describes the processes for the preparation of polished, fragmented gDNA, ligation of linkers, and removal of small fragments.

This chapter requires that the library amplification linkers are annealed, diluted to a working concentration of 500μM, and have passed quality control standards (Chapter 4). You will need 8μl of 500μM annealed linker stock per library preparation.

Step 1. Prepare the Sample and Polishing Master Mix

- 1. Bring the sheared DNA (20μg) to a volume of 80μl with 10mM Tris (pH 8.0) or VWR water in a 1.5ml tube.
- 2. Prepare the Polishing Master Mix in a 1.5ml tube. The amount of reagents needed for one reaction is listed below:

Polishing Master Mix	
10X NEB T4 DNA Polymerase Buffer (NEB2)	12μl
VWR water	9μl
100X NEB BSA	1μl
25mM dNTP working stock (made by mixing 100μl each of 100mM dA, dC, dT, and dGTP)	5μl
100mM ATP (ribonucleotide)	1μl
3U/μl T4 DNA Polymerase	6μl
10U/μl T4 Polynucleotide Kinase	6μl
Total	40μl

- 3. Pipette the Polishing Master Mix up and down 4 times to mix.

4. Aliquot 40µl of Polishing Master Mix to each sample (80µl post-fragmentation volume) for a total volume of 120µl per reaction.

Component	
Sample	80µl
Polishing Master Mix (from Step 1.1)	40µl
Total	120µl

5. Pipette the DNA plus Polishing Master Mix up and down 5 times to mix.

Step 2. Incubate the Polishing Reaction

1. Ensure that the plate seal or tubes lids are sealed. A heated lid is necessary because of the elevated temperature during the heat-kill.
2. Incubate in a thermocycler:
 - 20 minutes at 12°C
 - 20 minutes at 25°C
 - 20 minutes at 75°C

Note: Samples can be stored at 4°C overnight.

3. Transfer 1µl from each tube into a 0.2ml strip tube and save for QC purposes. Use 1 strip tube per sample genome.

Step 3. Ligate Linkers

1. To 119µl of polished DNA (from Step 2.3), add 8µl of 500µM annealed linker stock (from Chapter 4, Step 2.4).

Component	
Polished DNA (from Step 2.3)	119µl
500µM annealed linker stock (from Chapter 4, Step 2.4)	8µl
Total	127µl

2. Vortex and spin down the polished DNA + linkers.

3. Prepare a Ligation Master Mix for each sample library:

Ligation Master Mix	
10X NEB Buffer 2	8µl
100mM ATP (ribonucleotide)	4µl
VWR water	51µl
T4 DNA Ligase	10µl
Total	73µl

4. Add 73µl of the Ligation Master Mix to each tube of polished DNA + linkers for a total volume of 200µl per reaction.

Note: Add the Ligation Master Mix last to avoid excessive primer dimer formation.

Component	
Polished DNA + Linkers (from Step 3.1)	127µl
Ligation Master Mix (from Step 3.3)	73µl
Total	200µl

5. Pipette DNA, Linkers, and Ligation Master Mix up and down 5 times to mix.

Step 4. Incubate the Ligation Reaction

1. Ensure that the plate seal or tubes lids are sealed. Because incubation temperatures will not exceed room temperature, a heated lid is not necessary.
2. Incubate the ligation reaction in a thermocycler at 25°C for 90 minutes.
3. Remove 1µl from each sample into the next well in the strip tube and save for QC purposes.

Step 5. Remove Small Fragments

1. Thirty minutes before use, remove AMPure Beads from 4°C and equilibrate to room temperature.
2. Following the ligation reaction, transfer each ligated sample into a 1.5ml tube.
3. Add 2µl of 10% Tween 20 (v/v) to each ligated sample. Mix by pipetting up and down. Measure the volume.

Note: The Tween 20 solution should be diluted on the same day or in advance and stored in individual aliquots at -20°C. Old or degraded Tween 20 solutions can result in bead aggregation and unsuccessful separation of beads from DNA. Aggregated beads also can adhere to tube walls, making the tube walls appear brown.

4. Bring each sample to 300µl by adding ~99µl of 1X TE (pH 8.0). Mix by pipetting up and down.
5. Add 0.7x volume (~210µl) of AMPure Beads to each sample, wiping the pipette tip before adding beads.

Important: Mix AMPure Beads before adding to each sample.

6. Vortex and incubate at room temperature for 10 minutes.
7. Spin down for 1 - 2 seconds to collect the beads off the sides of the tube.
8. Place the sample into Dynal MPC-S (sample aligned with magnet). Allow the solution to clarify to facilitate bead recovery. This could take up to 5 minutes.
9. Pipette the supernatant into a clean, labeled 1.5ml tube. Set aside and save until recovery of sample from beads has been verified (Chapter 5, Step 6).

Important: Only begin pipetting the supernatant once it is clear (i.e. not brown in color). Make sure not to touch the beads when pipetting. The library is now on the beads, so save the beads.

10. Add 500µl of 70% EtOH to each collected bead pellet.
11. Invert twice in Dynal MPC-S. Remove and discard the EtOH by pipetting. Be sure the tube's lid is free of EtOH.
12. Add 500µl of fresh 70% EtOH to each collected bead pellet.

13. Invert twice in Dynal MPC-S. Remove and discard the EtOH by pipetting. Be sure the tube's lid is free of EtOH.

14. With lids open, place the Dynal MPC-S that holds the tubes into a 37°C incubator for 3 - 5 minutes to allow EtOH to volatilize.

Note: The beads should dry in Dynal MPC-S and appear “cracked” and completely dry. The use of a magnifying glass helps to determine whether the beads are “cracked.”

15. To elute library, add 80µl of VWR water to each tube and vortex for 10 seconds to resuspend the beads.

Note: The bead pellet is large. Ensure that the beads are fully resuspended (especially tiny dark brown specks of clumped beads). Library molecules will remain on the beads if the beads are not fully resuspended. If you see brown or stained tube walls and/or highly clumped beads typical of bead aggregation, keep the beads in the VWR water, close the tubes and place them in an air incubator at 37°C overnight. By morning the beads will have fully resuspended and can be efficiently isolated from your library using the Dynal MPC-S.

16. Place sample back in Dynal MPC-S and wait until the beads are cleared from the supernatant. Pipette the supernatant into a clean 1.5ml tube. If necessary, pipette any dried beads from the tube's walls to ensure maximum DNA recovery.

Important: Only begin pipetting the supernatant once it is clear (i.e. not brown in color). Make sure not to touch the beads when pipetting. The library is now in the supernatant, so do not discard it.

17. Remove 1µl from each tube and place into the next well in the strip tube to save for QC purposes.

Step 6. Evaluate the Sample

1. Measure the concentration of each sample using the NanoDrop spectrophotometer and record the measurements.

- Nebulized samples typically yield ~130ng/µl ($\pm$ 20ng/µl); 34% mean recovery
- Sonicated samples typically yield ~90ng/µl ($\pm$ 40ng/µl); 24% mean recovery

***Note:** The higher variance observed with sonication is a result of samples occasionally having a mode molecular weight of $\leq 300\text{bp}$. When the median fragment size approaches 300bp , $\sim 50\%$ of the fragments are $< 300\text{bp}$ and are therefore removed by the AMPure beads. Careful optimization and monitoring of the sonication protocol is necessary to avoid poor sample yield.*

- 2.** Remove 100ng of each sample and place into a 1.5ml tube. This will be used as template in Chapter 6, LM-PCR on Non-Captured Samples.

***Important:** It is highly recommended to perform LM-PCR on this sample (Chapter 6) before proceeding with Step 6.3 below, so that an aliquot of the reaction can also be included on the Bioanalyzer DNA Chip 7500 for analysis. This process will help to verify the efficacy of the linker ligation reaction.*

- 3.** Run an Agilent Bioanalyzer 2100 DNA Chip 7500 with the reserved $1\mu\text{l}$ aliquots:
 - Polishing (Step 2.3)
 - Ligation pre-size exclusion beads (Step 4.3)
 - Ligation post-size exclusion beads (Step 5.17)
 - (Optional) $1\mu\text{l}$ of LM-PCR product from Chapter 6, as recommended in Chapter 5, Step 6.2.

***Note:** Run at least one blank lane per DNA chip. The final library should be larger than 300bp and smaller than 700bp , and there should be no evidence of linker dimers ($\sim 40\text{bp}$ peak). Typically linker dimers will be present in ligation pre-size exclusion beads but not present in the blank lane (refer to Figure 6).*

- 4.** Calculate the total mass available for hybridization of each library (concentration $\times$ $48\mu\text{l}$ total volume remaining). A minimum of $5\mu\text{g}$ is recommended to proceed.
- 5.** Pipette $5\mu\text{g}$ of each library into a 1.5ml microfuge tube in preparation for hybridization (Chapter 7).

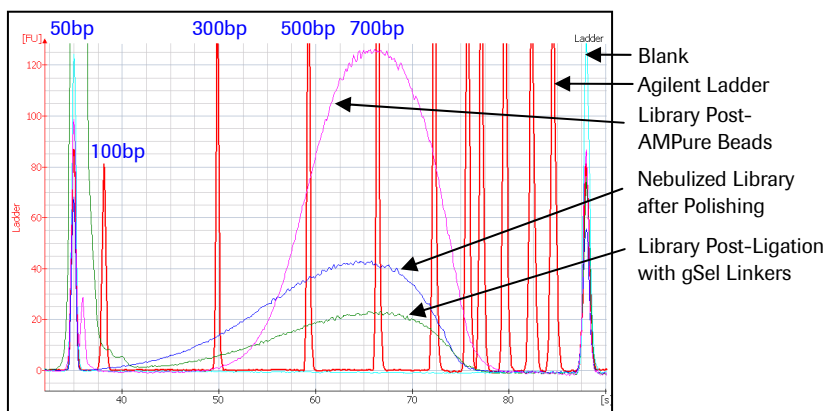


Figure 6: Example Agilent Bioanalyzer 2100 DNA 7500 Chip Run

The peaks include:

- Blank
- Agilent Ladder: 50*, 100*, 300*, 500*, 700*, 1,000, 1,500 ... 10,380
* These peaks are labeled in the ladder trace for reference purposes only.

- Library post-AMPure Beads.

Note: Only a small amount of small fragments remains, gSel dimer is removed, and the mode of library fragments produced (broad peak) is at ~700bp.

- Nebulized library after polishing
- Library post-ligation with gSel linkers

Note: Linker dimer peak is at ~40bp.

Notes

Chapter 6. LM-PCR on Non-Captured Samples

Chapter 6 describes how to amplify DNA samples that have not been captured using the linkers ligated in Chapter 5. In addition to verifying the efficacy of the linker ligation reaction, the amplification products will be used in Chapter 11 to estimate the magnitude of enrichment obtained in your Sequence Capture experiment. Roche NimbleGen highly recommends that this step is completed before proceeding to the hybridization step as a QC measure of the linker ligation.

References

- Thermocycler Manual
- Sigma Centrifuge Manual (Sigma-Aldrich)
- QIAquick PCR Purification Kit (Qiagen)

Step 1. Prepare the LM-PCR Master Mix

1. Dilute the PfuTurbo DNA Polymerase to 0.05U/ μ l and place on ice:

Component	
VWR water	44 μ l
10X ThermoPol Reaction Buffer	5 μ l
2.5U/ μ l PfuTurbo DNA Polymerase	1 μ l
Total	50 μ l

2. Prepare the LM-PCR Master Mix in a 1.5ml tube. The amount of each reagent needed for one sample (4 PCR reactions) is listed below:

LM-PCR Master Mix for 4 reactions (1 sample)

10X ThermoPol Reaction Buffer	20µl
25mM dNTP	2µl
40µM gSel3 Oligo*	5µl
VWR water	65µl
5U/µl Taq DNA Polymerase	4µl
0.05U/µl PfuTurbo DNA Polymerase	4µl
Total	100µl

* Desired 40µM working stock = 2µl of 4,000µM gSel3 stock + 198µl VWR water

Note: This protocol calls for the use of gSel3 primers rather than LMPCR3 primers for the amplification of captured DNA. The difference is that gSel3 primers contain thymine where LMPCR3 primers contain uracil at the corresponding nucleotide positions. The thymine-containing gSel3 primers are the same as used to construct adapters for Sequence Capture library construction (Chapter 4).

- Adjust volume of 100ng linker-ligated DNA set aside after library preparation (Chapter 5, Step 5.17) to 100µl using VWR water. Divide into 4 PCR tubes, each containing 25µl of DNA.
- Pipette 25µl of LM-PCR Master Mix into each reaction. Mix by pipetting up and down.

Step 2. Perform PCR Amplification

- Place PCR tubes in the thermocycler.
- Amplify samples using the following LM-PCR program:
 - Step 1: 2 minutes @ 95°C
 - Step 2: 1 minute @ 95°C
 - Step 3: 1 minute @ 60°C
 - Step 4: 2 minutes @ 72°C
 - Step 5: Go back to Step 2 and repeat 30 times
 - Step 6: 5 minutes @ 72°C
 - Step 7: Forever @ 4°C
- Store samples at 4°C until ready for cleanup.

Step 3. Clean up the Sample

1. Transfer each PCR reaction into a separate 1.5ml tube.
2. Follow the instructions provided with the QIAquick PCR Purification Kit with slight modifications (Steps 3.3 - 3.8 below).
3. To each tube, add 5 volumes (250µl) of Buffer PB from the QIAquick PCR Purification Kit. Mix well by pipetting up and down 5 times. Pipette into a QIAquick PCR Purification Kit column.
4. Centrifuge at 13,000rpm for 1 minute. Discard the flow-through.
5. Add 750µl of Buffer PE to the column. Centrifuge at 13,000rpm for 1 minute. Discard the flow-through.
6. Spin column at 13,000rpm for 1 minute to remove residual ethanol.
7. Add 50µl of VWR water or Buffer EB directly to the column matrix, changing tips between samples. Transfer the column to a 1.5ml microfuge tube. Centrifuge at 13,000rpm for 1 minute to elute the DNA.
8. Combine the 4 tubes for each sample into 1 tube, resulting in ~200µl total volume.

Step 4. Determine the Sample Concentration

1. Measure A_{260}/A_{280} using a NanoDrop spectrophotometer to quantify the concentration of DNA in the sample and to determine sample quality. Samples should fall within the following ranges:
 - Concentration: > 100ng/µl
 - A_{260}/A_{280} : 1.7 - 2.0

2. If samples meet the sample requirements, proceed to sample hybridization of the *unamplified*, linker-ligated library (Chapter 7).

Note: LM-PCR products are not hybridized to the microarray. Save them for use in qPCR analysis (Chapter 11).

If samples do not meet the sample concentration requirement, the sample ligation or LM-PCR may have failed. Repeat the library preparation process (Chapter 5) with new sample to ensure successful ligation and LM-PCR.

If samples do not meet the A_{260}/A_{280} requirement, purify using a second QIAquick PCR Purification column (Qiagen).

Caution: Do not hybridize these LM-PCR products to the microarray. If LM-PCR yield is low, stop! Proceed to Appendix A, Troubleshooting Sequence Capture Library Construction.

Chapter 7. Microarray Hybridization

Chapter 7 describes the NimbleGen protocol for sample hybridization. Be aware of the following:

- The hybridization protocol requires a NimbleGen Hybridization System. Refer to its user's guide for specific instructions on its use.
 - The hybridization protocol requires adherence of a NimbleGen mixer to the microarray slide.
 - 385K and 2.1M microarrays require different mixers, different hybridization volumes, and different amounts of Cot-1 DNA. Details of these differences are provided in Appendix D, step 2.
-

Reference

NimbleGen Hybridization System User's Guide (Roche NimbleGen)

Step 1. Prepare the Hybridization System

1. Set the Hybridization System to 42°C. With the cover closed, allow at least 3 hours for the heat block temperature to stabilize at 42°C.
 2. Turn on one heat block to 95°C and another to 70°C, and let equilibrate.
-

Step 2. Prepare the Hybridization Cocktail

1. Add 100µl (for 385K arrays) or 300µl (for 2.1M arrays) of 1mg/ml Cot-1 DNA to the sample from Chapter 5, Step 6.4 (5µg of fragmented, linker-ligated material).

2. Close each tube's lid and make a hole in the top of each tube's cap with an 18 - 20 gauge or smaller needle.

Note: The hole in the top of each tube's cap is a precaution to suppress contamination in the SpeedVac.

3. Dry the samples in a SpeedVac on high heat (60°C).

Note: This step may take 30 minutes or longer. To minimize drying time, dry Cot-1 DNA ahead of time and then add sample when ready.

Note: Denaturation of the DNA with high heat is not problematic after linker ligation because the hybridization utilizes single-stranded DNA.

4. Add 4.8µl (for 385K arrays) or 11.2µl (for 2.1M arrays) of VWR water to each sample to rehydrate.
5. Vortex the sample and centrifuge at maximum speed for 30 seconds.
6. Place sample in 70°C heat block for 10 minutes to fully solubilize the DNA.
7. Vortex sample and centrifuge at maximum speed for 30 seconds.
8. Add to each sample:
 - 8µl (for 385K arrays) or 18.5µl (for 2.1M arrays) of 2X SC Hybridization Buffer
 - 3.2µl (for 385K arrays) or 7.3µl (for 2.1M arrays) of SC Hybridization Component A

The sample should now contain the following components:

Component	385K Array	2.1M Array
Cot-1 DNA [1mg/ml]	100µl	300µl
VWR water	4.8µl	11.2µl
2X SC Hybridization Buffer	8µl	18.5µl
SC Hybridization Component A	3.2µl	7.3µl
Total	116µl	337µl

9. Vortex samples and centrifuge at maximum speed for 30 seconds.
10. Place each sample in a 95°C heat block for 10 minutes to denature the DNA.
11. Centrifuge samples at maximum speed for 30 seconds and place at 42°C until ready for hybridization (hybridize within 15 minutes of denaturation).

Step 3. Prepare Mixers

1. Remove the NimbleGen X1 (for 385K arrays) or HX1 (for 2.1M arrays) mixer from its package.

Note: For best results, use a NimbleGen Compressed Gas Nozzle to blow compressed gas across the mixer and slide to remove any dust or debris. Load samples within 30 minutes of opening the vacuum-packaged mixer to prevent the formation of bubbles during loading and/or hybridization.

2. Position the Precision Mixer Alignment Tool (PMAT) with the hinge on the left and then open it.
3. Snap the mixer onto the two alignment pins on the lid of the PMAT with the tab end of the mixer toward the inside hinge and the mixer's adhesive gasket facing outward (Figure 7).
4. Place the slide in the base of the PMAT while pushing back the plastic spring so that the barcode is on the right and facing up and the corner of the slide sits against the plastic spring. Remove your thumb and make sure the spring is engaging the corner of the slide and the entire slide is registered to the edge of the PMAT to the rightmost and closest to you. In addition, be sure that the slide is lying flat against the PMAT.

Note: Take care to align the slide correctly in the PMAT.

5. Gently blow compressed gas across the mixer and slide.
6. Remove the backing from the adhesive gasket using forceps and close the lid of the PMAT so that the gasket makes contact with the slide.

7. Lift the lid by grasping the long edges of the PMAT while simultaneously applying pressure with a finger through the window in the lid of the PMAT to free the mixer-slide assembly from the pins.

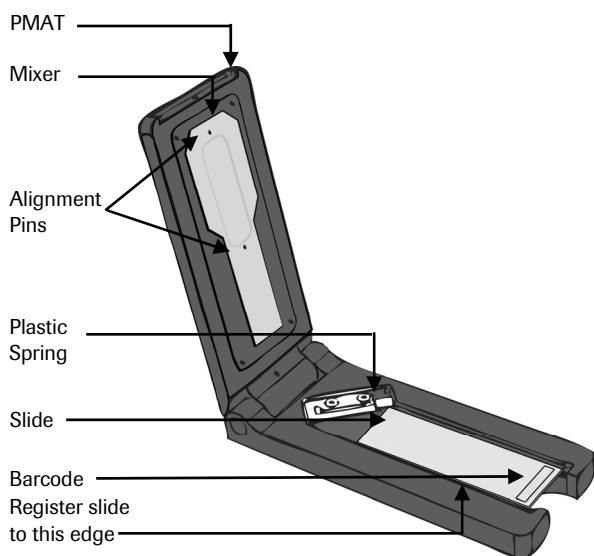


Figure 7: PMAT with X1 Mixer and Slide

8. Remove the mixer-slide assembly from the PMAT. Place the mixer-slide assembly on a clean, smooth, dark, flat surface.
Note: Place the mixer-slide assembly on the back of a 42°C heat block to facilitate adhesion of the mixer to the slide.
9. Rub the Mixer Brayer over the mixer with moderate pressure to adhere the adhesive gasket and remove any bubbles. Start in the center of the array and rub outwards. The adhesive gasket will become clear when fully adhered to both surfaces.
10. Repeat Steps 3.1 - 3.9 for all slides to hybridize.
11. Place the mixer-slide assembly in the slide bay of the Hybridization System.

Step 4. Load and Hybridize Samples

1. Confirm that each sample is matched with the appropriate microarray.

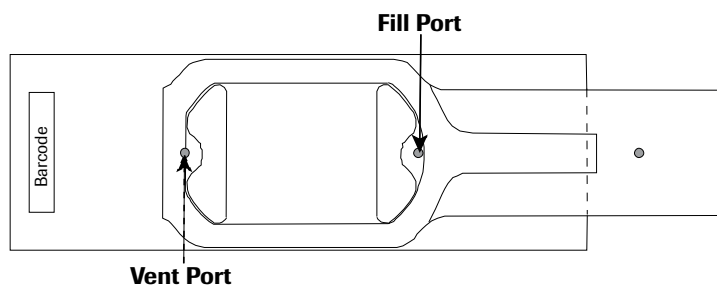


Figure 8: X1 Mixer and Slide

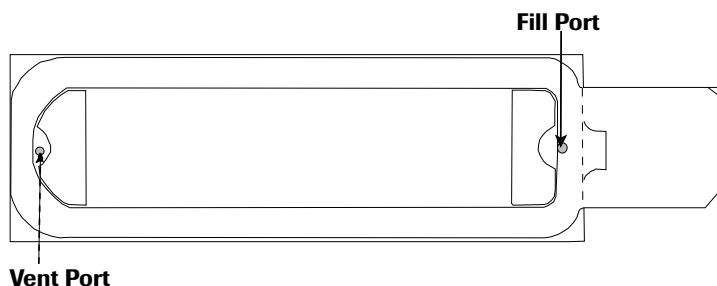


Figure 9: HX1 Mixer and Slide

2. Use a Microman 100 Pipette with CP100 tips (Gilson) to pipette as much of the sample as necessary to completely fill the mixer chamber without introducing any bubbles (~15 - 16 μ l for the 385K array and X1 mixer or ~34 - 37 μ l for the 2.1M array and HX1 mixer) into the fill port. After loading, make sure there are no bubbles in the mixer chamber. If there are, gently massage bubbles to either of the ends of the mixer chamber or to the sides. Pipette any extra sample into the port (where there is no bubble) and remove bubbles out through the opposite port.
3. Dry any exposed sample from the fill and vent ports using a clean tissue or cotton swab for each array to avoid cross-contamination.

4. Adhere port seals to cover the fill and vent ports on the mixer. Press on both the vent and fill port stickers at the same time to ensure equal pressure.
5. Close the bay clamp until locked (you should hear a click).
6. Turn on the Mixing Panel and set the mix mode to B on the Hybridization System.
7. Confirm the system recognizes the slide in each occupied bay.
8. Approximately 5 - 10 minutes after starting the Hybridization System:
 - Ensure that the mix mode is set to B.
 - Ensure that the green light is displayed for all occupied stations.
 - Ensure that the temperature reads 42°C.
9. Hybridize samples at 42°C for at least 64 hours (no more than 72 hours).

Chapter 8. Assembly of Elution Chamber

Chapter 8 describes the processes for assembly of the Elution Chamber. It is essential that the Elution Chambers are assembled before beginning Chapter 9, Microarray Washing and Elution of Captured Samples.

Be sure to select the correct Elution Chamber for the type of array you are using in the experiment: 385K arrays require Elution Chambers *ES1* and 2.1M arrays require Elution Chambers *EL1*. Elution Chambers and Gasket are disposable. Use once and discard to avoid cross-contamination between samples.

Step 1. Inspect the Elution Chamber Components

1. While wearing gloves, in a workplace in which gSel-adapted genomic DNA has **not** been manipulated (e.g. laminar fume hood), remove the clear polycarbonate Elution Insert from its plastic bag.

Note: Elution Chambers should not be exposed to gSel-linkered libraries or LM-PCR amplified DNA since such molecules could cross-contaminate samples.

2. Remove one Elution System Gasket for each Elution Chamber to be assembled from the shipping bag.
3. Visually examine the Elution System Gasket to make sure it is intact.

Note: The Elution System Gasket has a square cross-sectional shape with a tiny groove on each face of the square. Visual examination of the groove during assembly identifies twists in the Elution System Gasket during assembly.

Step 2. Assemble the Elution Chamber

1. Wearing gloves, insert the Elution System Gasket into the oblong channel of the molded Elution Insert, starting with the two straight portions of the channel.

2. While holding down the center portion of the Elution Insert, work the Elution System Gasket into one side of the rounded part of the channel (Figure 10).
3. Fit the Elution System Gasket into the remaining rounded part of the channel while still holding down the center portion of the Elution Insert (Figure 10).
4. Gently press the Elution System Gasket down into the channel to ensure proper, uniform Elution Chamber assembly (Figure 10).

Important - for 2.1M arrays only: The Elution Chamber EL1 has a significantly larger chamber area and Gasket than those in the Elution Chamber ES1 pictured in Figure 10.

Note: Do **not** twist the Elution System Gasket in any way. Visual inspection should reveal the same face of the square facing up. Trace the path of the tiny groove on the Elution System Gasket once it is in the channel. A twist in the Elution System Gasket could result in a poor seal to the array face resulting in a leaky elution.

Important: The Elution System Gasket needs to be evenly distributed within the Elution Insert channel to be sure that its entire length is accommodated.

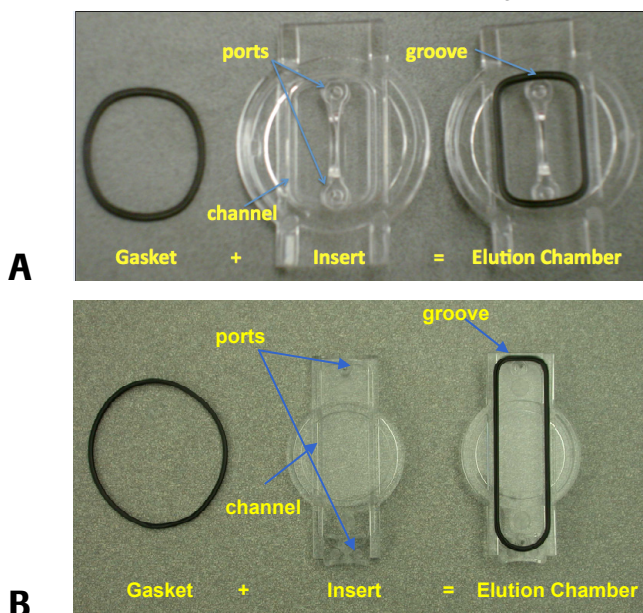


Figure 10: Assembly of an Elution Chamber ES1 (A) and an Elution Chamber EL1 (B) Using the Elution System Gasket and the Elution Insert

Chapter 9. Microarray Washing and Elution of Captured Samples

Chapter 9 describes the processes for single tube microarray washing and elution of the captured gDNA samples for batches of 6 slides or fewer. These processes are prone to sample cross-contamination, which can be amplified by downstream PCR steps. Take appropriate precautions. Prepare single-use wash tubes for each sample. Discard tubes after one use.

Note: It is extremely important that the water bath temperature is closely monitored and remains at 47.5°C. A high quality thermometer placed in the water bath is recommended since the displayed temperatures on many water baths are often imprecise.

Note: Equilibrate buffers @ 47.5°C at least 2 hours before washing microarray.

Step 1. Prepare for Wash and Elution

1. Dilute 10X SC Wash Buffers (I, II, and III) and 2X Stringent Wash Buffer to 1X working solutions.
2. For each slide, prepare the following SC Wash Buffers:
 - Two washing tubes with 32ml of Stringent Wash Buffer heated to 47.5°C in a water bath
 - One washing tube with 32ml of SC Wash Buffer I at room temperature
 - Two washing tubes with 32ml of SC Wash Buffer II: one at 47.5°C and one at room temperature
 - One disassembly basin (100ml multichannel pipettor basin) with 100ml SC Wash Buffer II heated to 47.5°C

Note: Recent array hybridization data suggest that significant inter-array variation is caused by temperature fluctuations that can occur during the transfer between the 42°C NimbleGen Hybridization System and the 47.5°C SC Wash Buffer II in the disassembly basin. Therefore, this step could be among the most critical in the procedure. Make sure arrays are kept at a constant 42°C throughout the hybridization step and that they are immediately transferred, one at a time, to wash buffer in the disassembly basin temperature maintained at 47.5°C for mixer disassembly.

- One washing tube with 32ml of SC Wash Buffer III at room temperature
- 3.** Prepare 125mM sodium hydroxide (NaOH). The following volumes are appropriate for processing single arrays. Adjust volumes accordingly if processing multiple arrays:
- For 385K arrays: Mix 493.75µl VWR water and 6.25µl 10M NaOH.
 - For 2.1M arrays: Mix 987.5µl VWR water and 12.5µl 10M NaOH.
 - Vortex to mix, spin down briefly, and store at room temperature until use.
- 4.** For each array to be eluted, fill a separate 1.5ml tube with the appropriate volume of 125mM NaOH at room temperature.
- For 385K arrays: 425µl of 125mM NaOH
 - For 2.1M arrays: 900µl of 125mM NaOH
- 5.** Prepare 20% acetic acid:
- 400µl VWR water
 - 100µl acetic acid, glacial
 - Vortex to mix, spin down briefly, and store at room temperature until use.

Note: Prepare fresh 125mM NaOH and 20% acetic acid solutions each time prior to use.

Step 2. Disassemble and Wash Microarrays

1. Double-glove both hands (double-gloving facilitates easy removal of the outer gloves between each sample). Change gloves between each sample to avoid cross-contamination.
2. Remove the slide from the 42°C Hybridization System and place the slide directly into the disassembly basin containing 100ml of SC Wash Buffer II heated to 47.5°C.
3. Wait ~10 seconds for the temperature to equilibrate. Carefully peel the mixer off the slide.

Note: Handle the slide only by the edges and portions exposed before mixer removal. Never touch the surface of the slide inside the mixer area. Agitate the slide up and down for 5 seconds in SC Wash Buffer II.

Important: The mixer is extremely flexible. Peel the mixer off slowly to avoid breaking the slide. Due to the larger footprint of the HX1 mixer, special caution is required in removing HX1 mixers from 2.1M microarrays. Practicing with extra HX1 mixers and blank slides is recommended.

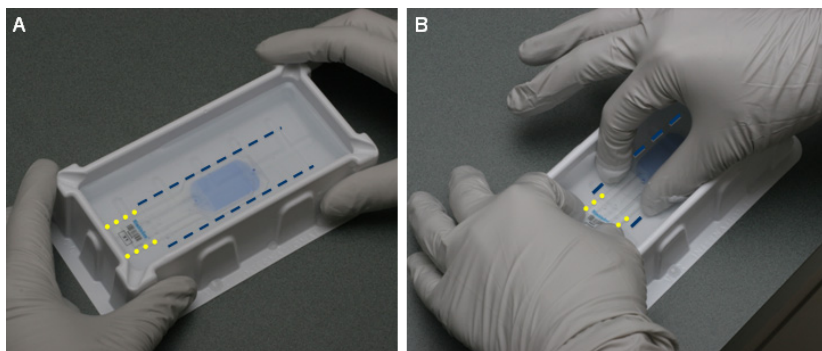


Figure 11: Disassembly of X1 Mixer from Slide. (A) Note the front edge of the mixer (dotted lines) and the edges of the slide (dashed lines). (B) Depiction of how the edges of the slide and the edges of the mixer should be handled to properly remove the mixer from the slide. For visualization purposes only, this figure depicts the mixing chamber of the mixer (both A and B) loaded with dye.

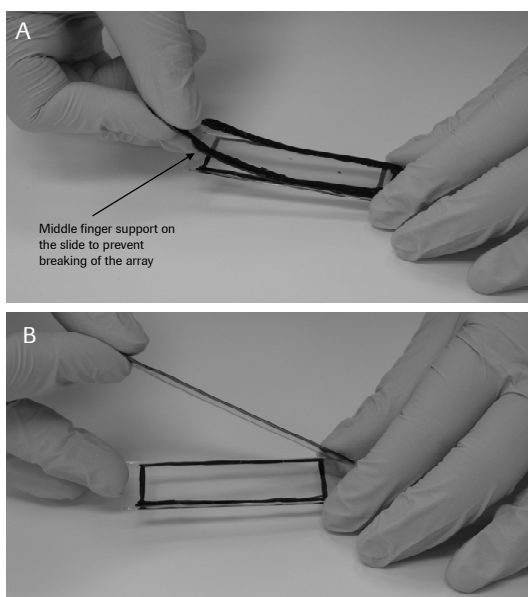


Figure 12: Disassembly of HX1 Mixer from Slide. *Note: Mixer removal is performed while the mixer-slide assembly is submerged in 100ml of 47.5°C SC Wash Buffer II. The mixer-slide assembly has been removed from the disassembly basin and the edges of the HX1 mixer are outlined in marker here for visualization purposes only. (A) The slide is supported by the middle finger (noted by arrow) to prevent breaking of the 2.1M array, and the HX1 mixer is pulled off the slide by peeling upward. (B) Depiction of the HX1 mixer completely disassembled from the slide.*

4. Transfer the slide to a wash tube containing 47.5°C SC Wash Buffer II, fix the cap, and invert the tube 10 times at a rate of 1 inversion per second.

Important - for 2.1M arrays only: Since the 2.1M array is larger and closer to the edge of the slide compared to a 385K array, extra care should be taken to only touch the edges of the slide when handling with gloves and forceps.

5. Remove the outer gloves and replace with new gloves.
6. Change gloves and repeat Steps 2.2 - 2.5 until all slides have been disassembled, using new disassembly basins, wash tubes, and SC Wash Buffers for each slide to be disassembled. To ensure that the SC Wash Buffer II does not cool below 47.5°C, we recommend that disassembly of multiple slides is performed in batches of ≤ 6 .

Important: *If multiple slides will be disassembled and washed, it is important to ensure that one Elution Station is available for each slide in the batch so that no slides remain in the final wash buffer longer than necessary while waiting for an available Elution System.*

7. Using new, disposable forceps for each slide to be handled, quickly and carefully remove each of the slides from their wash tubes containing SC Wash Buffer II. Place slides in new wash tubes containing 32ml of 47.5°C Stringent Wash Buffer (1 slide per tube).
8. Invert the tubes 10 times at a rate of 1 inversion per second. Place in the tube rack for 5 minutes at 47.5°C.
9. Remove the tubes from 47.5°C incubator. Invert the tubes 10 times at a rate of 1 inversion per second.
10. Using new, disposable forceps for each slide to be handled, quickly and carefully remove each of the slides from their wash tubes containing Stringent Wash Buffer at 47.5°C.
 - Place slides in new wash tubes containing 32ml of 47.5°C Stringent Wash Buffer (1 slide per tube).
11. Invert the tubes 10 times at a rate of 1 inversion per second. Place in the tube rack for 5 minutes at 47.5°C.
12. Remove the tubes from 47.5°C incubator and invert the tubes 10 times at a rate of 1 inversion per second.
13. Using new, disposable forceps for each slide to be handled, quickly and carefully remove each of the slides from their wash tubes containing Stringent Wash Buffer at 47.5°C.
 - Place slides in new wash tubes containing 32ml of SC Wash Buffer I at room temperature (1 slide per tube).
14. Invert the tubes at a rate of 1 inversion per second for 2 minutes.
15. Using new, disposable forceps for each slide to be handled, quickly and carefully remove each of the slides from their wash tubes containing Wash I.
 - Place slides in new wash tubes containing 32ml of SC Wash Buffer II at room temperature (1 slide per tube).
16. Invert the tubes at a rate of 1 inversion per second for 1 minute.

17. Using new disposable forceps for each slide to be handled, quickly and carefully remove the slides from the wash tubes containing SC Wash Buffer II.
 - Place slides in new wash tubes containing 32ml of SC Wash Buffer III at room temperature (1 slide per tube).
18. Invert the tubes 10 times at a rate of 1 inversion per second.

Important: Avoid prolonged exposure of the slide in SC Wash Buffer III before elution.

Step 3. Elute the DNA

Roche NimbleGen recommends that the following procedure be performed as quickly as possible.

Note: Perform the following steps at room temperature. Place the Elution System in a heat block that is turned off or on a bench top. If multiple arrays are being eluted simultaneously, assemble each one in an Elution System as quickly as possible to prevent DNA from eluting into SC Wash Buffer III.

1. Remove the slides from the wash tubes using your gloved fingers. Gently tap the end of the slide on a clean tissue to remove as much excess buffer as possible.

Note: Be careful not to tap too hard and break the slide.

2. Wipe the *back* side of the slide with a clean tissue (the *front* side of the slide is the side on which you can read the barcode numbers in the correct orientation). Place the slide *front* side up within the Elution System at room temperature (Figure 13).

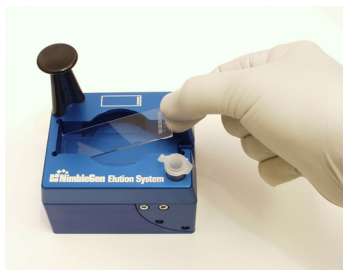


Figure 13: Placing a Slide into the Elution System

3. Place the disposable Elution Chamber on top of the slide with the Elution System Gasket facing down (Figure 14).
 - For 385K arrays: Use Elution Chamber ES1 and Gasket.
 - For 2.1M arrays: Use Elution Chamber EL1 and Gasket.



Figure 14: Placing the Elution Chamber in the Elution System

4. Place an Elution System Retaining Ring over the Elution Chamber and lock into place (Figure 15).



Figure 15: Placing and Locking the Elution System Retaining Ring

5. Using its handle, tilt the top of the Elution System until it locks into the upright position. Slowly pipette the freshly made 125mM NaOH into the bottom (filling) port of the Elution Chamber (Figure 16).
 - For 385K arrays: Pipette ~425 μ l of the 125mM NaOH solution into the elution chamber. Return any excess 125mM NaOH that does not fit in the elution chamber to the original 1.5ml tube.
 - For 2.1M arrays: Pipette ~900 μ l of the 125mM NaOH solution into the elution chamber. Return any excess 125mM NaOH that does not fit in the elution chamber to the original 1.5ml tube.

Return the Elution System to the horizontal position.

Important: Do **not** remove the pipette tip from the filling port until you have returned the Elution System to the horizontal position or the 125mM NaOH will leak out.

Important: Do **not** tilt the Elution System without a pipette tip placed in the filling port or the 125mM NaOH will leak out (Figure 16).

Note: Pipette the 125mM NaOH solution slowly so it does not come out the top (vent) port.

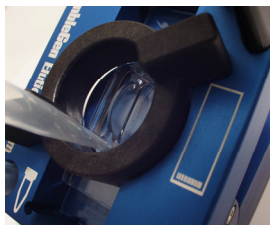


Figure 16: Tilting the Elution System and Loading Sample into the Elution Chamber

6. Incubate at room temperature for 10 minutes.
7. After incubation, pipette eluted DNA from the Elution Chamber and place into a 1.5ml tube containing any extra volume of 125mM NaOH that did not fit into the Elution Chamber.

Disassemble and discard the Elution Chamber.

Note: The Elution System can be tilted to an upright position, and a pipette can be used to remove any remaining volume from the slide and to transfer it to the 1.5ml tube containing the eluted DNA.

Note: Do not re-use arrays or Elution Chambers because linked DNA will be carried over from the previous experiment. Observe all national, regional, and local regulations for proper disposal of microarrays and Elution Chambers.

8. Add 20% acetic acid solution to the Qiagen Buffer PBI in a 1.5ml tube.
 - For each 385K array: Mix 16µl 20% acetic acid with 500µl Buffer PBI.
 - For each 2.1M array: Prepare two 1.5ml tubes containing 16µl 20% acetic acid with 500µl Buffer PBI.

Pipette up and down 10 times to mix.

9. Add 20% acetic acid/Buffer PBI mixture to eluted samples:

- For 385K arrays: Add the entire ~425µl eluate to the 1.5ml tube containing the 516µl of 20% acetic acid/Buffer PBI. Pipette up and down 10 times to mix.
- For 2.1M arrays: Divide the ~900µl eluate evenly between two tubes each containing 516µl 20% acetic acid/Buffer PBI. Pipette up and down 10 times to mix.

Note: The color of the eluate/acetic acid/PBI should be yellow. If the color is purple, add 20% acetic acid, 1µl at a time, to the DNA mixture until the color changes to yellow.

- 10.** Prepare the vacuum manifold and MinElute column following the supplier's instructions. Transfer the DNA/PBI/acetic acid mixture to a single MinElute column on the vacuum manifold. Draw the solution through the column and release the vacuum. Note that the maximum capacity of a column is 750µl, so multiple loadings will be required to transfer the entire volume of each sample to a single MinElute column.

- For 385K arrays: Use a single MinElute column.
- For 2.1M arrays: Use a single MinElute column.

Note: Roche NimbleGen strongly recommends the use of a vacuum manifold, instead of a microcentrifuge, for the purification of DNA using the Qiagen MinElute columns. The use of a vacuum manifold may decrease the chance of contamination between samples when multiple samples are purified simultaneously. Refer to the Qiagen MinElute Handbook for the following steps.

- 11.** Add 750µl of Buffer PE (containing EtOH per the manufacture's instructions) to the column on the vacuum manifold. Draw the solution through the column and release the vacuum.
- 12.** Place the MinElute column into a 1.5ml tube or 2ml collection tube and centrifuge at maximum speed for 1 minute to remove any residual Buffer PE. Discard the flow-through.
- 13.** Place the MinElute column in a clean 1.5ml tube. Add 50µl Buffer EB to the center of the column, wait 1 minute, and centrifuge at maximum speed for 1 minute.

14. Adjust the volume of each eluate to 320µl using VWR water.
15. Proceed to Chapter 10, LM-PCR on Captured Samples process, or keep the eluate at -20°C until use.

***Note:** Due to the increased amount of captured DNA recovered from an array when the NaOH elution process is used, it is necessary to utilize the LM-PCR protocol described in Chapter 10 when amplifying the captured DNA.*

Chapter 10. LM-PCR on Captured Samples

Chapter 10 describes the amplification of eluted, captured DNA samples using LM-PCR.

References

- Thermocycler Manual
 - QIAquick PCR Purification Kit Instructions (Qiagen)
-

Step 1. Prepare the LM-PCR Master Mix

1. Prepare the LM-PCR Master Mix in a 1.5ml tube. The amount of each reagent needed for one reaction is listed below. Twelve reactions are recommended for amplification of *each* captured sample and 4 reactions for amplification of a no-template (negative) control (refer to Step 1.3):

Note: The increased amount of LM-PCR primer used in this protocol is necessary to compensate for the higher yield of DNA obtained when using the NaOH Elution method.

LM-PCR Master Mix	Amount
10X ThermoPol Reaction Buffer	4.9µl
25mM dNTP	0.5µl
40µM gSel3 ¹	6.25µl
VWR water	11.35µl
5U/µl Taq DNA Polymerase	1µl
0.05U/µl PfuTurbo DNA Polymerase ²	1µl
Total	25µl

1 Desired 40µM gSel3 working stock = 2µl of 4,000µM gSel3 stock + 198µl VWR water

2 For 0.05U/µl working stock, dilute 2.5U/µl PfuTurbo DNA Polymerase 1:50 in 1X ThermoPol Reaction Buffer (refer to Chapter 6, Step 1.1).

2. Briefly vortex the master mix.
3. Aliquot 25µl of DNA from Chapter 9, Step 3.14 into PCR tubes (or 96-well PCR plate).
 - For captured DNA sample: Add 25µl of undiluted DNA directly to 12 PCR tubes (or wells).
 - For negative control sample: Add 25µl VWR water to 4 PCR tubes (or wells).
4. Add 25µl of LM-PCR Master Mix to each PCR tube (or well). Mix by pipetting up and down.

Step 2. Perform PCR Amplification

1. Place PCR tubes (or 96-well plate) in the thermocycler.
2. Amplify samples using the following LM-PCR program:
 - Step 1: 2 minutes @ 95°C
 - Step 2: 1 minute @ 95°C
 - Step 3: 1 minute @ 60°C
 - Step 4: 2 minutes @ 72°C
 - Step 5: Go back to Step 2 and repeat 27 times
 - Step 6: 5 minutes @ 72°C
 - Step 7: Forever @ 4°C

***Note:** This LM-PCR on Captured Samples program uses 27 cycles, which is different than the 30 cycles used for LM-PCR on Non-Captured Samples (Chapter 6). Be careful to use the correct program.*

3. Store samples at 4°C until ready for cleanup.
-

Step 3. Clean up the Sample

1. Transfer each PCR reaction into a separate 1.5ml tube.
2. Use the QIAquick PCR Purification Kit for cleanup of the PCR reactions. Follow the instructions provided with the kit with slight modifications (Steps 3.3 - 3.8 below).

***Note:** To maximize recovery of LM-PCR product, only 1 LM-PCR reaction should be purified per column. Thus, for each LM-PCR amplified captured DNA sample (12 individual PCR reactions) you will use 12 QIAquick columns for the clean up of the DNA.*

3. To each tube, add 5 volumes (250µl) of Buffer PB from the QIAquick PCR Purification Kit. Mix well by pipetting up and down 5 times. Pipette into a QIAquick PCR Purification Kit column.
4. Centrifuge at 13,000rpm for 1 minute. Discard the flow-through.
5. Add 750µl of Buffer PE to the column. Centrifuge at 13,000rpm for 1 minute. Discard the flow-through.
6. Spin column at 13,000rpm for 1 minute to remove residual ethanol.
7. Add 50µl of VWR water or Buffer EB directly to the column matrix, changing tips between samples. Transfer the column to a 1.5ml microfuge tube. Centrifuge at 13,000rpm for 1 minute to elute the DNA.
8. Combine the 12 tubes for each sample into 1 tube, resulting in ~600µl total volume.
9. Measure A_{260}/A_{280} to quantify the concentration of DNA in the sample and to determine sample quality (Step 4).

Step 4. Determine the Sample Concentration

1. Measure A_{260}/A_{280} using the NanoDrop spectrophotometer to quantify the concentration of DNA in the sample and to determine sample quality. Samples should fall within the following ranges:

- Total Yield: $> 5\mu\text{g}$
- A_{260}/A_{280} : 1.7 - 2.0

2. If samples meet the minimum requirements, proceed to Chapter 11, qPCR on LM-PCR Amplified Samples.

If samples do not meet the minimum yield requirement, sample ligation or LM-PCR on captured DNA sample may have failed. New samples should be put through library preparation (Chapter 5) to ensure successful linker ligation and LM-PCR.

If samples do not meet the A_{260}/A_{280} requirement, purify using additional QIAquick columns.

Chapter 11. qPCR on LM-PCR Amplified Samples

Chapter 11 describes the qPCR assays used to estimate relative fold-enrichment. These assays are an inexpensive way to determine whether the capture was successful prior to sequencing.

A standardized set of qPCR SYBR Green assays are employed as internal quality controls for NimbleGen Sequence Capture experiments performed with human total gDNA. The genomic loci recognized by these assays are included as capture targets on every NimbleGen Human Sequence Capture array. Comparison by qPCR of the relative DNA concentrations of these control loci in non-captured and captured samples allows for the estimation of enrichment of a capture target before committing sample libraries to expensive and/or time-consuming downstream applications.

The internal control region assays recommended in this chapter were selected due to their convenience for use across different species and because they produce consistent results. It is worth considering designing and evaluating locus specific qPCR assays for your own region(s) of interest because capture results can vary from locus to locus. It is possible that your regions of interest may enrich differently than our internal control regions.

For more information regarding general PCR and qPCR methods, consult the PCR Applications Manual (3rd Edition), available from the Roche Applied Science website (www.roche-applied-science.com).

Step 1. Perform Advance Preparations

1. Determine the number of DNA samples to be analyzed.

***Note:** A “DNA sample” in this chapter is defined as one non-captured LM-PCR product (Chapter 6) and the matching captured LM-PCR product (Chapter 10). Assuming a standard set of 4 NimbleGen Sequence Capture (NSC) control locus qPCR assays will be used and qPCR assays will be performed in triplicate, each captured DNA sample will require 24 individual qPCR reactions for analysis. One no-template-control (NTC) should always be included to monitor for contamination in qPCR assay primers, other qPCR reagents, and the entire PCR process. One positive control template, ideally consisting of the original genomic DNA starting material, should always be included to verify assay function. The NTC and positive controls will each require 12 additional qPCR reactions (refer to Figure 17).*

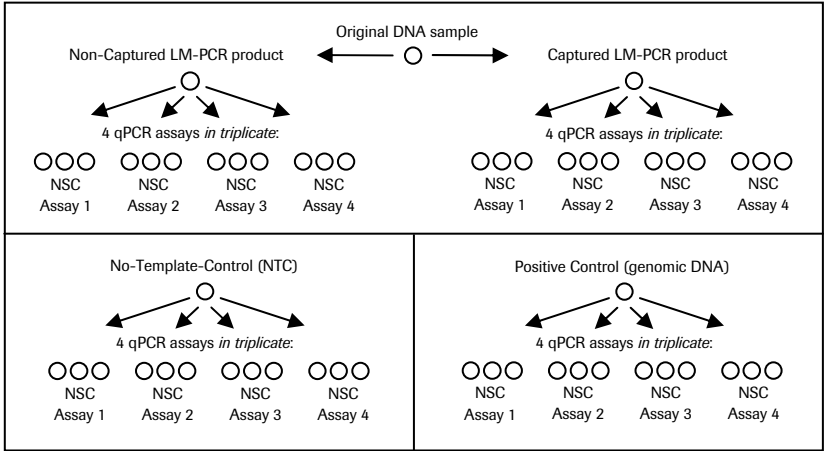


Figure 17: qPCR Experimental Overview

2. The number of reaction wells required to perform a complete control locus qPCR analysis is determined by the formula, $W = 24 \times (S + 1)$, where W is the total number of wells required and S is the number of original DNA samples in the experiment (one per array). For example, analyzing one captured DNA sample, one NTC, and one positive control with 4 NSC assays performed in triplicate requires 48 individual qPCR reactions, $W = 24 \times (1 + 1)$. Analyzing two captured DNA samples requires 72 qPCR reactions, $W = 24 \times (2 + 1)$, etc.

3. Dilute the NSC assay forward and reverse primers to 2μM (see Table 2, Step 1.6 on next page).
4. Dilute sufficient amounts of non-captured and captured LM-PCR products to a concentration of 5ng/μl in PCR-grade water for use as qPCR templates (~100μl each).

***Note:** These LM-PCR products are used as templates in the control locus qPCR reactions. The initial concentration of these products was previously determined (Chapter 6, Step 4.1 and Chapter 10, Step 5.1).*

***Important - for users of multi-array sets:** If you are performing Sequence Capture using a multi-array set, and decided to pool your multiple library preparations to increase their uniformity prior to hybridization (Appendix C), you also will have pooled the non-captured LM-PCR products into a single tube. Note that captured LM-PCR products should **not** have been pooled.*

5. Add the following for a 15μl NSC assay (for larger volume reactions, adjust the reagent amounts proportionally):

Component		
	PCR-grade water	5.9μl
	NSC Assay forward primer (2μM)	0.3μl
	NSC Assay reverse primer (2μM)	0.3μl
	SYBR Green Master (2X)	7.5μl
	5ng/μl template (non-captured LM-PCR, captured LM-PCR, or positive control genomic DNA)	
	–or–	1μl
	VWR water (no-control template)*	
	Total	15μl

* The final concentration of templates in the reaction should be 0.333ng/μl except for the no control (VWR water) template.

6. Program the qPCR instrument using the conditions specified in Table 1.

***Note:** These conditions are optimized for use with the LightCycler 480 Instrument II and LightCycler 480 SYBR Green I 2X Master Mix. The use of a different thermocycler or reagents could require altering these conditions to achieve optimal results.*

Table 1. qPCR instrument cycling conditions

Program Name	Cycles	Analysis Mode	Target	Hold	Ramp	Acquisitions (per °C)	Acquisition mode
			(°C)	(hh:mm:ss)	Rate (°C/s)		
Pre-incubation	1	None	95	00:10:00	4.8	---	None
Amplification	40	Quantification	95	00:00:10	4.8	---	None
			60	00:01:00	2.5	---	Single
Melting Curve	1	Melting Curves	95	00:00:10	4.8	---	None
			65	00:01:00	2.5	---	None
			95	---	---	5	Continuous
Cooling	1	None	40	00:00:10	2	---	None

Table 2. Recommended NSC qPCR Assays. Forward and reverse primer sequences for 4 different NSC control locus assays are shown in Table 2. All of these assays have been validated on NimbleGen Sequence Capture arrays using human captured DNA. The use of these assays for analyzing captured DNA from other species has not been tested and is not currently supported by Roche NimbleGen.

NSC qPCR Assay Name	Primer Sequences (5' → 3')	T _m (°C)	Product Length	qPCR Efficiency (E)
NSC-0237	F: CGCATTCTCATCCCAGTATG R: AAAGGACTTGGTGCAGAGTTTCAG	81.15	80bp	1.84
NSC-0247	F: CCCACCGCCTTCGACAT R: CCTGGCTTACTGTGGGCTCTTG	81.03	74bp	1.80
NSC-0268	F: CTCGCTTAACCAGACTCATCTACTGT R: ACTTGGCTCAGCTGTATGAAGGT	78.99	75bp	1.78
NSC-0272	F: CAGCCCCAGCTCAGGTACAG R: ATGATGCGAGTGCTGATGATG	82.23	71bp	1.93

Control Locus NSC qPCR assays enable you to measure the enrichment of a small set of standardized capture control loci that represent a range of known capture efficiencies. These assays act as a proxy for estimating the enrichment of larger populations of capture targets without a need for sequencing. If qPCR analysis using NSC assays indicates a successful capture of the control loci, it is likely that the experimental loci of interest were also successfully captured.

It is recommended that the 4 assays listed in Table 2 are the minimum number used for analysis of NimbleGen Sequence Capture arrays to obtain an adequate representation of the diverse population of capture target loci. However, analysis using additional assays that are custom designed by you to measure enrichment of your specific targets of interest could provide additional useful information.

Step 2. Set up Control Locus qPCR Reactions

1. Using the information provided in Chapter 11, Steps 1.1 - 1.3, set up the required number of NSC assays with non-captured LM-PCR templates, captured LM-PCR templates, NTCs, and positive control templates (gDNA). Set up the reactions inside a PCR hood to minimize contamination.
2. Run the reactions in the thermocycler using the cycling conditions listed in Table 1.
3. Following raw data collection, run the Absolute Quantification Analysis Module within the LightCycler 480 Basic software using the 2nd Derivative Maximum Method. It is not necessary to run a template standard curve for the analysis unless you want to measure the efficiency of the qPCR assays. The raw CP values will be used to perform a simplified version of *relative* quantification comparing non-captured and captured DNA samples.
4. Perform a melting curve (dissociation) analysis to verify that nonspecific amplification products, primer dimers, and other artifacts are not contributing to the CP values for any samples.
5. Copy or export the CP values to a spreadsheet program for further analysis.

***Note:** CP (crossing point) values reported by the LightCycler 480 Instrument II and software are analogous to the C_t (crossing threshold) values reported by other instruments and represent the cycle at which fluorescence signal in a reaction well rises above background fluorescence signals in that well. The CP value measured for a sample is dependent on the initial concentration of template DNA in the reaction. Lower CP values correspond to higher initial template concentrations.*

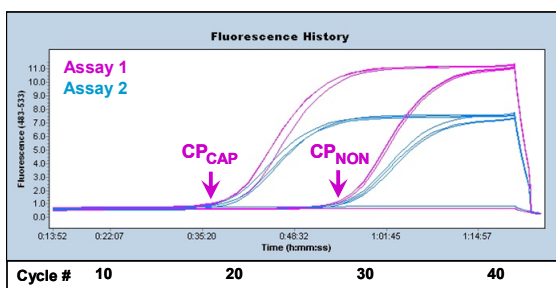


Figure 18: Example of Sequence Capture qPCR Data for Two NSC Assays Generated Using the LightCycler 480 Instrument II. In a successful experiment, the CP values from qPCR of captured LM-PCR templates (CAP) will be significantly lower than CP values from non-captured templates (NON) for all assays.

Step 3. Analyze Data

1. Calculate the average CP values for all replicate reactions.

Note: Replicate CP values obtained from qPCR of post-LM-PCR samples should be similar. If the calculated standard deviation for 3 replicate CP values is > 0.2 for a qPCR assay, consider repeating that assay to obtain more consistent results.

2. Confirm that the NTC reactions did not produce significant fluorescent signals, which might indicate a problem with PCR contamination resulting in difficulty interpreting experimental results.

Note: Primer dimer amplification can occasionally produce an increase in fluorescence signal in an NTC reaction in the absence of target template molecules, but this typically occurs very late in the run (i.e. CP approaching 35 cycles), and it is easy to differentiate primer dimer from target amplicon by melting curve analysis (refer to Step 2.4). Primer dimers are typically indicated by a peak melting temperature significantly lower than the peak melting temperature of the correct amplicon. Primer dimers in an NTC reaction are not usually a concern because they are an artifact of low-affinity annealing in the absence of competing template. However, fluorescence created from the presence of primer dimer (as indicated by a second, lower T_m peak in the melting curve) in an experimental sample reaction can lead to an erroneously high estimate of template concentration in that sample, and sample(s) with multiple melting curve peaks should be regarded as suspect and omitted from CP value determinations.

3. For each different sample and NSC assay combination, subtract the average CP value measured for the captured LM-PCR template from the average CP value measured for the matching non-captured LM-PCR template. This value is the delta-CP. A successfully enriched captured sample should generate a lower CP value than its matched non-captured sample. Thus the delta-CP calculated from an NSC assay should be positive if the capture process enriched the corresponding locus.
4. Calculate the fold-enrichment for a NSC control locus by raising the PCR Efficiency (E) for that assay to the power of the delta-CP measured for the corresponding control locus, or $E^{\text{delta-CP}}$.

Note: When PCR assays operate at 100% theoretical efficiency (i.e. a perfect doubling of target sequences in every cycle), $E = 2$. The E values for the NCS assays listed in Table 2 were measured by Roche NimbleGen. However, because multiple parameters (PCR instrument, reagent lots, etc.) can affect the efficiency of a PCR assay, it is recommended to determine E values empirically in your own laboratory for each different NSC assay. The efficiency of NSC assays is determined in the same way as other PCR assays by applying linear regression analysis to amplification data from a template standard dilution series. The slope of the standard curve is used to determine E with the equation $E = 10^{(-1/\text{slope})}$. A slope of -3.3 indicates an E value of 2. Calculated E values less than 2 are common. The software provided with most qPCR instruments can perform calculations of E automatically, or they may be calculated using external mathematical or spreadsheet software.

Example: NSC-0268 assay (assuming $E = 1.78$)

- Replicate CP values for non-captured, post-LM-PCR sample = 28.3, 28.5, 28.4
- Replicate CP values for captured, post-LM-PCR sample = 17.5, 17.3, 17.7
 - Average $CP_{\text{non-captured}} = 28.4$
 - Average $CP_{\text{captured}} = 17.5$
 - Delta-CP = 10.9
 - Fold enrichment ($E^{\text{delta-CP}} = (1.78)^{10.9} = 537$

Example: NSC-0272 assay (assuming $E = 1.93$)

- Replicate CP values for non-captured, post-LM-PCR sample = 28.0, 28.2, 28.1
- Replicate CP values for captured, post-LM-PCR sample = 18.2, 18.1, 18.0
 - Average $CP_{\text{non-captured}} = 28.1$
 - Average $CP_{\text{captured}} = 18.1$
 - Delta-CP = 10
 - Fold enrichment ($E^{\text{delta-CP}} = (1.93)^{10} = 717$

Note: Average CP values for no-template-control (NTC) assays should be negligible, or they may indicate the presence of cross-contamination among wells or reagent contamination. If this is observed, repeat the qPCR experiment.

Note: Average CP values for positive control (genomic DNA) assays should be similar (within ~1 CP) to the average CP values obtained for non-captured, post-LM-PCR samples.

Data Analysis Considerations

Careful consideration and data interpretation are necessary when deciding whether to sequence captured samples. Consider the types of targets and how the control loci represent them, the region targeted for enrichment, and qPCR as an estimation platform.

The large difference between the fold enrichment values for the two example NSC assays (above) demonstrates the importance of testing multiple NSC control loci to obtain values more likely to accurately represent the larger population of capture targets. This difference is primarily influenced by two parameters: 1) differences in capture efficiency of the different loci, and 2) amplification efficiency differences between the two qPCR assays. Without determination of the efficiency of the qPCR assays in each run, the local differences in capture efficiency cannot be determined.

The efficiency of the qPCR assays can be variable across runs, which means that strict determination requires a standard dilution curve for each locus during each run. This user's guide assumes that across a collection of loci, both the intrinsic locus capture efficiencies and the individual qPCR reaction efficiencies will settle in upon some common mean value. Therefore, employments of more loci yield better global estimates. It is not uncommon for highly efficient qPCR assays to demonstrate fold enrichment values on the order of 1,000 or more. However, benchmarking with such large values results in over-estimating global

capture (i.e. pulls the average fold enrichment up too far). Also, platform error in qPCR measurements is $\pm 50\%$ (or one cycle when comparing two measures) meaning that it takes many replicates to discriminate a 300-fold enrichment from a 600-fold enrichment. Consideration of this platform error means that 2-fold differences should be considered the same measure, rather than different measures of enrichment. Consider the size of your sequencing budget and whether to optimize NimbleGen Sequence Capture designs iteratively. Then establish qPCR benchmarks that fit your needs.

The average maximum enrichment level of capture targets within a large genome is dependent on the size of the capture target as a percentage of its genome. For example, if 50% of a genome were targeted for capture, an ideal result should yield no better than a 2-fold average enrichment for the targeted loci. Smaller capture targets in the same genome would have higher maximum average enrichment levels. Thus, the definition of a “successful” NimbleGen Sequence Capture experiment, as estimated by fold-enrichment values for control loci, might differ substantially with different sized capture targets or different downstream applications for the captured DNA.

Notes

Appendix A. Troubleshooting Sequence Capture Library Construction

This appendix describes how to troubleshoot the failure of LM-PCR on non-captured samples. Two main causes for this type of failure are library construction failure (molecules not ligated with linkers) or PCR failure.

LM-PCR failure on captured samples (but successful on non-captured samples) most likely represents a problem with microarray hybridization, washing, or elution.

Failure of LM-PCR on Non-Captured Samples

To evaluate the robustness of the library construction process:

1. To two aliquots of 2μl each quality controlled linkers, add 78μl of VWR water (this represents the post-fragmentation volume in Chapter 5, Steps 1.1 - 1.3).
2. Label the first aliquot as “test.” Process this sample as described in Chapter 5, Steps 1.1 - 4.3.
3. Label the second aliquot as “enzymatic negative control.” Process this sample the same as the “test” sample (Chapter 5, Steps 1.1 - 4.3), except add VWR water instead of the enzymes listed in Step 1.1 and Step 3.1.

Important: For both samples, do not perform small fragment removal (Chapter 5, Steps 5.1 - 5.16).

4. Load 20μl (18μl of sample + 2μl of 10X loading dye) onto a 4% agarose gel (1X TAE or 0.5X TBE). Evaluate using ethidium fluorescence. An alternative to gel analysis is to use the Bioanalyzer DNA 1000 Kit.

The gSel linker complexes contain a blunt end suitable for ligation to the 3' end of gSel3 (5' end of gSel4) with a recessed 5' end on gSel3 that needs to be “filled out” to the 3' end of gSel4 (by two bases) by T4 DNA Polymerase.

Be aware of the following:

- A failure to “fill-out” will result in linker dimer formation during ligation. Since the gSel complex is 20bp, a 40bp product will be present upon complete ligation of a failed “fill-out” (Figure 19, - T4 pol, lanes 8, 9, and 10).
- A failure at the T4 polynucleotide kinase reaction step will leave the filled out ends with only a 5' phosphate on gSel4 (where it was synthesized) but not on gSel3. Hence, the ligation will not be effective since only one strand may be covalently linked by the ligase. Consequently, a ladder pattern will form with the most intense bands at 20bp with higher molecular weight bands fading out (Figure 19, - T4 PNK, lanes 11, 12, and 13).
- A failure at the ligation step will result in only 20bp products (Figure 19, - ligase, lanes 14, 15, and 16).
- A successful Sequence Capture library should result in a 20bp ladder pattern with the most intense bands near the top of the gel, and fading at the bottom (Figure 19, +++, lanes 5, 6, and 7). If the agarose gel resembles lanes 5, 6, and 7, the library construction reagents are working properly.

***Note:** Addition of all library construction enzymes should result in long polymers of double-stranded linker complexes since the construction process polishes ends and makes them competent.*

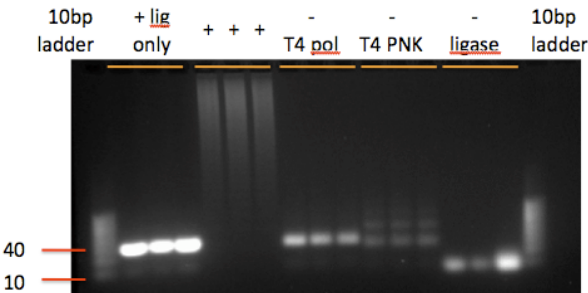


Figure 19: Troubleshooting the Failure of LM-PCR on Non-Captured Samples Using Different Positive and Negative Conditions by 4% Agarose Gel Electrophoresis

5. Replace library construction reagents based upon which set of lanes your reactions most resemble. If the lane for the test sample looks like the lane for the enzymatic negative control sample, replace all library construction reagents. If the lane for the test sample resembles lanes 5, 6, and 7 (Figure 19, +++), the LM-PCR reaction should be suspected as the cause for failure.

An alternative to agarose gel electrophoresis is to run reactions on a Bioanalyzer 2100 using the DNA 1000 Kit (Figure 20).

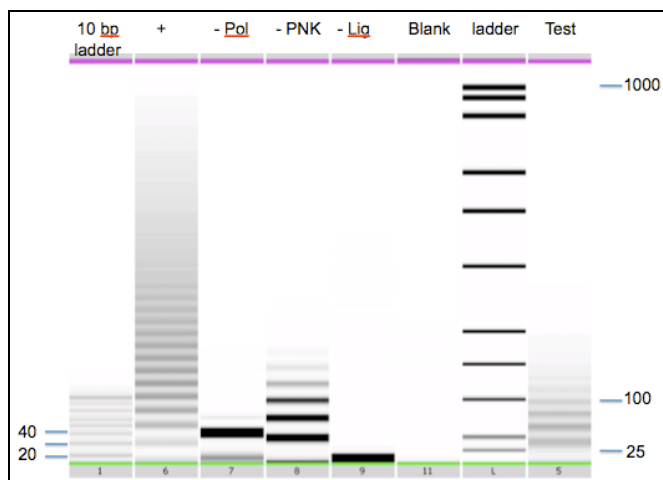


Figure 20: Troubleshooting the Failure of LM-PCR on Non-Captured Samples Using Different Positive and Negative Conditions by Use of the Bioanalyzer DNA 1000 Chip

Notice that the construction reagents used for the Test sample yielded a result that resembled the result for the - PNK sample. This resemblance suggests that the kinase reaction was only partially successful.

Failure of LM-PCR on Captured Samples

Contact Roche Microarray Technical Support for help troubleshooting failure of LM-PCR. Refer to page 10 for contact information.

Notes

Appendix B. Sequencing with Next Generation Platforms

This appendix describes important aspects to consider when sequencing NimbleGen Sequence Capture libraries using next generation platforms.

***Important:** Before working with sequencing reads, it is necessary to remove the gSel linker sequences (refer to Chapter 1 for the linker sequences). Most sequence assembly programs have methods for trimming vector sequence, which can be used to accomplish this task.*

Recommended Procedure: 454 GS FLX Titanium Series Sequencing

Reference

GS FLX Titanium General DNA Library Preparation Method Manual (April 2009 version)

***Note:** Instructions for sequencing NimbleGen Sequence Captured Libraries with 454 GS FLX Standard chemistry have been discontinued in favor of the Titanium method.*

The Sequence Capture library has been amplified and is now ready for DNA Library preparation. For GS FLX Titanium chemistry, library construction requires 3 - 5µg of captured library for the double SPRI method option and 5 - 10µg of captured library for the gel cut method option. To maximize sequencing performance, the quality of the resulting 454 library should fall within the parameters found in the GS FLX Titanium General Library Preparation Method Manual (April 2009 version).

Process: GS FLX Titanium Series

1. Evaluate the concentration of the amplified Sequence Capture library.
 - For the double SPRI method option, use 3 - 5µg of library and adjust total volume to 100µl with Qiagen EB buffer or with 1X TE. If the volume is > 100µl, use Qiagen QiaQuick column to concentrate library.
 - For the gel cut method option, use 5 - 10µg of library and adjust volume to 20µl. If the volume is > 20µl, use a Qiagen MinElute column to concentrate the library.
2. Using the GS FLX Titanium General DNA Library Preparation Method Manual, proceed to Section 3.2, Fragment Size Selection (page 21). Perform fragment size selection using the applicable protocol option.
3. Follow the remaining steps of the GS FLX Titanium Library Preparation Method Manual through Section 3.10, Library Quality Assessment and Quantitation.

Note: If you have technical questions regarding 454 sequencing, contact your Roche Sequencing Account Manager or Roche Sequencing Technical Support. Go to www.454.com/contact-us/technical-support.asp for contact information.

Alternative Procedure: Sequencing Using Short Read Technology Platforms

Considerations

- Short reads are defined for this appendix as ≤ 50 bp in length.
- The captured material has a gSel3 linker on the 5' end and its complement (gSel4-P_i) on the 3' end of each molecule. Sequencing the captured material directly results in considerable coverage of the linkers but generally reveals very little insert sequence.
- Sonication of the Sequence Capture library and ligation of platform-specific linkers will increase the probability of sampling the insert sequence with short read technologies. Because most molecules will break internally only once, sonicating small fragments (300 - 500bp) is not efficient. Sequencing from a library generated by this method results in a significant proportion of the sequencing reads (i.e. ~50%) attributed to the linkers: two ends of each fragment with the linker and two ends (the internal break) with the insert.
- The preferred method for reducing the proportion of linker complexes within the sequencing reads would entail catenation of the library inserts followed by sonication. The products from LM-PCR of the captured material should have blunt ends to be compatible with the Pfu polymerase used for amplification. Roche NimbleGen recommends repairing the ends of the library before ligating the inserts to each other. Treat the library as a genomic sample intended to be shotgun sequenced. Use the manufacturer's recommendation to perform sonication or nebulization of the catenated products and the subsequent ligation of platform-specific linkers.

Notes

Appendix C. Sequence Capture with Multi-Array Sets

This appendix describes issues pertaining to the use of multi-array sets for Sequence Capture of DNA samples. Suggested protocol modifications for using multi-array sets are detailed here and throughout the user's guide where applicable. Read this Appendix thoroughly before proceeding.

Considerations

- Some very large Sequence Capture targets (e.g. chromosome regions greater than 30Mb) may be divided among multiple arrays to provide sufficient coverage by oligonucleotide capture probes.
- Sequence Capture experiments using a multi-array set require proportionally more DNA starting material than experiments using a single array. For example, the amount of genomic DNA starting material required for Sequence Capture using a single microarray is 20µg, whereas for Sequence Capture using a five-array set is a minimum of 100µg.
- As with any large-scale molecular biology experiment, including experiments using multi-array sets, we recommend that you generate extra library at the beginning of your experiment (Chapters 2 - 6). This helps ensure that sufficient material will be available for hybridization to multi-array sets if there are low yields during library preparation, or if there is a need to repeat hybridization to one array in a set without repeating the full set.

For example, a Sequence Capture experiment using a 5-array set that starts with the minimum recommended amount of 100µg total genomic DNA (i.e. five 20µg library preparations), will often yield sufficient library for hybridization to 6 or more arrays. In some cases, additional library preparation(s) at the outset of the experiment (i.e. six 20µg library preparations) may help to ensure sufficient library yield. Your choice should take into account the amount of DNA available to you and your level of experience with the Sequence Capture protocol.

- When performing a Sequence Capture experiment using a multi-array set, we recommend you process your DNA sample as multiple independent 20µg preparations in parallel. Combining them into one scaled-up (100µg) preparation has not been tested.
- To minimize variation in enrichment level among the different arrays of a multi-array set, it may be helpful to ensure the library material is as uniform as possible prior to the hybridizations. This **optional** step can be accomplished by pooling multiple library preparations, mixing them, and splitting them again immediately prior to hybridization (see “Instructions for Pooling Library Preparations for Hybridization to Multi-Array Sets” below).

Note: Pooling as a means to increase uniformity prior to hybridization should only be done using library preparations generated from the same original genomic DNA sample.

Instructions for Pooling Library Preparations for Hybridization to Multi-Array Sets

Note: If you are processing multiple DNA preparations in parallel from the same original sample for hybridization to a multi-array set, and have decided to pool and mix your multiple library preparations to increase their uniformity prior to hybridization, follow the protocol provided below:

1. After having confirmed that each library preparation was successful by Bioanalyzer analysis (Chapter 5, Step 6) and LM-PCR analysis (Chapter 6), pool the libraries into one 1.5ml tube and mix by pipetting up and down.
2. Measure and record the concentration of the pooled library.
3. Calculate the total mass available for hybridization of each sample (concentration x total volume).

Important: You should have enough library to hybridize at least 5µg to each array in your multi-array set. If you have less than 5µg of library for each array, contact Roche Microarray Technical Support for additional guidance.

4. In preparation for hybridization, pipette 5µg of the pooled library into one 1.5ml microfuge tube for each array in the multi-array set.
5. Store any remaining library at -20°C. This library could be used to repeat a hybridization to one of the arrays in the multi-array set.

6. Pool the purified LM-PCR products that were generated in Chapter 6 into a single 1.5ml tube (these correspond to the library samples that were pooled earlier in this appendix). Mix by vortexing.
7. Measure A_{260}/A_{280} to quantify the concentration of DNA in the sample.
Note: Save a sufficient amount of purified LM-PCR product for use in qPCR analysis (i.e. “non-captured LM-PCR products,” Chapter 11).
8. Proceed to sample hybridization of the *unamplified*, linker-ligated library (Chapter 7).

Notes

Appendix D. Sequence Capture with 2.1M Arrays

This appendix describes protocol differences in performing Sequence Capture with 385K and 2.1M arrays. Where applicable, these differences are also noted throughout the text of this user's guide. Read this appendix thoroughly before proceeding.

Differences between 385K and 2.1M Sequence Capture Experiments

1. **Array size:** The size of the glass slide for 385K and 2.1M arrays is identical, but the area of the slide occupied by the oligonucleotide array is significantly different (refer to the following images). This difference requires more careful handling of 2.1M slides to avoid disturbing oligonucleotides near the edge of the array.

385K Microarray Slide



2.1M Microarray Slide



2. **Hybridization Cocktail (Chapter 7, Step 2):** Although 2.1M and 385K Sequence Capture experiments use the same amount of library DNA (5 μ g) for hybridization to the microarrays but different volumes for the hybridization solutions (Step 2.8, Component table).
3. **Hybridization Mixers (Chapter 7, Step 3):** Sequence Capture with 2.1M arrays requires a NimbleGen HX1 mixer (Figure 9) to accommodate the larger hybridization volume.

4. **Hybridization Volume (Chapter 7, Step 4.2):** Pipette up to 37 μ l of the sample hybridization mix into the fill port. 35 μ l of sample hybridization mix is usually sufficient to fill the mixer chamber.
5. **Assembly of Elution Chamber (Chapter 8):** Select the correct Elution Chamber for the type of microarray you are using in the experiment: Elution Chambers ES1 for 385K microarrays and Elution Chambers EL1 for 2.1M microarrays.
6. **Disassembly of Mixers from Microarrays (Chapter 9, Step 2):** Due to the larger footprint of the HX1 mixer, special attention is required in removing HX1 mixers from 2.1M microarrays. We recommend practicing this step with HX1 mixers and blank slides.
7. **Handling of 2.1M Slides During Washing Steps (Chapter 9, Step 2):**
Because the 2.1M array is larger and closer to the edge of the slide than the 385K array, take extra care when handling the slide with glove and forceps.
8. **Elution Volume and Handling of Elution Reagent (Chapter 9, Step 3):** The larger volume of an Elution Chamber EL1 requires the addition of more Elution Reagent (~900 μ l) than required by an Elution Chamber ES1 (~425 μ l).
***Important:** Do not remove the pipette tip from the filling port until you have returned the Elution System to the horizontal position or elution reagent will leak out.*
***Important:** Do not tilt the Elution System to the upright position until you have inserted the pipette tip into the filling port or elution reagent will leak out.*
9. **Prepare Eluted Samples for LM-PCR on Captured Samples (Chapter 10, Step 1).**

Appendix E. Alternative Elution Method Using Elution Reagent

This appendix describes the elution of captured DNA from arrays using the Elution Reagent, provided in the NimbleGen Sequence Capture Wash and Elution Kit. Less captured DNA might be eluted when using Elution Reagent instead of 125mM NaOH as recommended in Chapter 9.

Step 1. Prepare for Wash and Elution

1. Place a thermometer into the slot in the NimbleGen Elution System. Adjust the heat block until the thermometer reads at least 95°C (can take up to 1 hour).
 2. For each sample to be eluted, fill one (for 385K arrays) or two (for 2.1M arrays) 1.5ml tubes(s) with Elution Reagent and place the tube(s) in a 95°C heat block (tube slot is provided in the Elution System).
 3. Dilute 10X SC Wash Buffers (I, II, and III) and 2X Stringent Wash Buffer to 1X working solutions as described in Chapter 9, Step 1.4.
-

Step 2. Disassemble and Wash the Microarrays

Follow the instructions in Chapter 9, Step 2.

Step 3. Elute the DNA

Roche NimbleGen recommends that the following procedure be performed as quickly as possible.

Warning! *The NimbleGen Elution System is heated to 95°C. Once inserted into the Elution System, the Elution Chamber and slide become hot and may burn you if caution is not exercised.*

1. Remove the slides from the wash tubes using your gloved fingers. Gently tap the end of the slide on a clean tissue to remove as much excess buffer as possible.

Note: Be careful not to tap too hard and break the slide.

2. Wipe the *back* side of the slide with a clean tissue (the *front* side of the slide is the side on which you can read the barcode numbers in the correct orientation). Place the slide *front* side up within the 95°C Elution System (Figure 13).
3. Place the disposable Elution Chamber on top of the slide with the Elution System Gasket facing down (Figure 14).
4. Place an Elution System Retaining Ring over the chamber and lock into place (Figure 15).
5. Using its handle, tilt the top of the Elution System until it locks into the upright position. Slowly pipette ~425µl (for 385K arrays) or ~900µl (for 2.1M arrays) of 95°C Elution Reagent into the bottom (filling) port of the Elution Chamber (Figure 16).

Return the Elution System to the horizontal position.

Important: *Do not change the position of the elution system without a pipette tip placed in the filling port or the elution reagent will leak out.*

Note: *If multiple arrays are being eluted simultaneously, assemble each one in the Elution System as quickly as possible to prevent DNA from eluting into SC Wash Buffer III.*

6. Incubate at 95°C for 5 minutes. Pipette reagent back and forth across the array when bubbles form.

Note: Tilting the top of the Elution System aids in smooth mixing.

7. After incubation, pipette eluted DNA and place into a 1.5ml sample tube.
8. Repeat Steps 3.5 - 3.7.
 - If you are using 385K arrays, combine the elution volumes into one 1.5ml sample tube.
 - If you are using 2.1M arrays, a larger volume of Elution Reagent was used and combining two ~900µl volumes will exceed the capacity of the 1.5ml sample tube. Pipette the second elution (~900µl into a second 1.5ml sample tube.

- 9.** Rinse the array briefly with a third volume of Elution Reagent:
- For 385K arrays: ~425µl of 95°C Elution Reagent
 - For 2.1M arrays: ~900µl of 95°C Elution Reagent
- 10.** For 385K arrays, pipette the third elution volume out of the Elution Chamber and combine with the first two elution volumes.
- For 2.1M arrays, split this third elution of ~900µl into the two sample tubes already obtained to this point.
- 11.** Disassemble and discard the Elution Chamber.
- Note: The Elution System is heated to 95°C. The Elution Chamber and slide are also very hot and may burn you if caution is not exercised.*
- Note: Do **not** re-use arrays or Elution Chambers. Sample contamination can occur even after vigorous washing. Observe all national, regional, and local regulations for proper disposal of microarrays and Elution Chambers.*
- 12.** Close each tube's lid and make a hole in the top of each tube's cap with an 18 - 20 gauge or smaller needle.
- Note: The hole in the top of each tube's cap is a precaution to suppress molecules transferring between eluates in the SpeedVac.*
- 13.** Completely dry samples in a SpeedVac set to 60°C.
- Note: This drying step could take 2 hours or longer. Alternatively, dry the sample overnight in a vacuum concentrator set to 30°C.*
- Important - for 2.1M arrays only: The drying time may be slightly longer for 2.1M eluates due to the slightly larger volumes of Elution Reagent per sample tube.*
- 14.** Store samples at -20°C or proceed to Chapter 10, LM-PCR on Captured Samples. Before proceeding to LM-PCR, perform the following steps:
- a. Rehydrate eluted samples in 320µl of VWR water (for both 385K and 2.1M arrays). For 2.1M arrays, be sure to rehydrate the two tubes to a combined total of 320µl in a single 1.5ml storage tube.
 - b. Vortex samples and centrifuge at maximum speed for 3 seconds.
 - c. Incubate at 70°C for 10 - 12 minutes.
 - d. Vortex samples and centrifuge at maximum speed for 3 seconds.

Notes

Appendix F. Recommendations for High-Throughput Sequence Capture Experiments

This appendix provides recommendations for optimizing high-throughput Sequence Capture experiments when multiple arrays are processed simultaneously.

- Elute DNA samples using sodium hydroxide (NaOH). Because captured samples are eluted at room temperature, instead of 95°C, it is easier to process more arrays simultaneously as the researcher does not need to control for bubble formation in the elution chamber. Additionally, the use of Qiagen's vacuum manifold, instead of a microcentrifuge, for the purification of DNA may decrease the probability of cross-contamination when a large number of samples are purified simultaneously.
- Include additional negative controls throughout the process to monitor for potential contamination from the work environment, shared equipment, or shared reagents.
- Include both positive and negative controls when performing LM-PCR for captured and non-captured samples. For the negative control, 25µl of VWR water can be substituted for the DNA. This sample can then be purified following amplification and included in the qPCR reactions to further control for contamination of the reagents used for the LM-PCR setup. The positive control can be any previously captured or non-captured DNA sample that has amplified successfully in the past. This control enables the researcher to better evaluate the success of the LM-PCR reactions, as well as, identify potential contamination of samples.
- When purifying 96-well plates containing LM-PCR reactions, consider using the Qiagen QIAquick 96-well Purification Kit (Catalog No. 28181) for the simultaneous purification of up to 96 LM-PCR reactions rather than using single columns. The following, additional equipment is required for use with the QIAquick 96-well Purification Kit: Qiagen Centrifuge 4-16 (Catalog No. 81310) and Qiagen Plate Rotor 2x96 (Catalog No. 81031).

Notes

Appendix G. Limited Warranty

ROCHE NIMBLEGEN, INC. NIMBLEGEN ARRAYS

1. Limited Warranty

A. Products: Roche NimbleGen, Inc. ("Roche NimbleGen") warrants that its Products conform to its published specifications and are free from defects in material or workmanship. Customer's sole and exclusive remedy (and Roche NimbleGen's sole and exclusive liability) under this limited warranty shall be to either (a) replace the defective Products, or (b) provide Customer with a refund, as solely determined by Roche NimbleGen.

B. Under no circumstances shall Roche NimbleGen's liability to Customer exceed the amount paid by Customer for the Services and Products to Roche NimbleGen. Roche NimbleGen will bear all reasonable shipping costs if service is re-performed at Roche NimbleGen or the Products are replaced. This warranty does not apply to any defect or nonconformance caused by (i) the failure by Customer to provide a suitable storage, use, or operating environment for the Materials or Customer's submission of substandard quality Materials or contaminated or degraded Materials to Roche NimbleGen, (ii) Customer's use of non-recommended reagents, (iii) Customer's use of the Products, Materials or Data for a purpose or in a manner other than that for which they were designed, (iv) the failure by Customer to follow Roche NimbleGen's published protocols; or (v) as a result of any other abuse, misuse or neglect of the Products, Materials or Data by Customer. This warranty applies only to Customer and not to third parties.

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D. Any action by Customer against Roche NimbleGen for Roche NimbleGen's breach of this warranty must be commenced within 12 months following the date of such breach. Notwithstanding such 12-month period, within twenty (20) days of the delivery of Data and/or Products to Customer, Customer must notify Roche NimbleGen in writing of any nonconformity of the Services and Products, describing the nonconformity in detail; otherwise all Services and Products shall be conclusively deemed accepted without qualification.

2. FURTHER LIABILITY LIMITATION

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